Impact of Toll-like Receptors on Epstein-Barr Virus and Burkitt's Lymphoma

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The only aids used for composing this dissertation are those stated therein.

Zurich, October 2009
Ludwig Zauner

Love is the only rational act.

– Stephen Levine

To my family

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Summary

Epstein-Barr virus (EBV) infects over 90% of the human population and persists in the infected individual lifelong without further complications. However, EBV is associated with several malignancies including Burkitt's lymphoma (BL), which is endemic in the Sub-Saharan African region and affects children around 5 years of age. Nevertheless, the contribution of EBV to the development of BL remains elusive. Other factors in addition to EBV have been identified, e.g., the malaria pathogen *Plasmodium falciparum*. *P. falciparum* was shown to have multiple effects on the human innate immune system including the triggering of the germ line encoded Toll-like receptor 9 (TLR9). Importantly, as ligands of TLR9 were shown to affect EBV-induced transformation, we aimed to investigate the effect of TLRs on EBV-associated malignancies.

The work presented here focused on the impact of innate immunity (TLRs) towards EBV. We hypothesized that triggering TLR9 inhibits EBV lytic gene expression and thereby reduces the probability that EBV will undergo reactivation from latency. As latency is a prerequisite for transformation, a clear understanding of the factors that regulate lytic infection is critical. To investigate TLR9 stimulation, its subsequent signaling pathways, and its effects on EBV in more detail, we used EBV-positive BL cell lines as a model system. Expression of TLRs and the effects of TLR triggering on cell growth and cytokine expression were assessed. Most importantly, signaling pathways triggered by TLR9 were investigated, with a focus on the regulation of the switch between lytic and latent EBV.

The data presented here provide new insights concerning the regulation of EBV gene expression in the context of TLR engagement. This markedly contributes to the understanding of how EBV and chronic innate immune stimulation, as seen in malaria, may affect BL development by forcing EBV to stay latent. Most importantly, our data argue that acetylation and phosphorylation on histones is the key step in the TLR9-induced suppression of EBV lytic gene expression. The novelty of how TLR9 affects viral promoter activation will be of great benefit for evaluating the role of TLR9 in cancer onset and development.

Zusammenfassung

Das B-lymphotropische Epstein-Barr Virus (EBV) findet sich bei über 90% der humanen Weltbevölkerung wider. Nach Primärinfektion etabliert EBV eine lebenslang andauernde latente Infektion in den Gedächtnis B-Zellen des Wirtes. Unter bestimmten Bedingungen entwickelt sich die latente Infektion zu einem EBV-assoziierten Tumor wie zum Beispiel dem endemischen Burkitt's Lymphom (BL), welcher vor allem in Afrika südlich der Sahara bei Kindern um die 5 Jahren auftritt. Welchen Einfluß EBV bei der Tumorentstehung jedoch hat, ist ungewiss. Eine Rolle in der Entstehung des BL könnten weitere Faktoren spielen: das Malaria Pathogen *Plasmodium falciparum* stimuliert auf mehrfache Weise das angeborene Immunsystem, insbesondere den Toll-like Rezeptors 9 (TLR9). Unhabhängig davon wurde gezeigt, dass die Aktivierung von TLR9 einen Einfluß auf die *in vitro* Transformation durch EBV hat. Aufgrunddessen richteten wir unsere Untersuchungen auf die Auswirkungen von TLRs in Bezug auf EBV-assoziierte Tumoren.

Die hier dargestellte Arbeit konzentriert sich auf die Konsequenzen der TLR-aktivierten Signalwege bezüglich der Genexpression von EBV. Da EBV in latenter Phase eine Vorraussetzung für die Transformation der Zelle darstellt, ist ein Verständnis der Faktoren welche die lytische Phase regulieren von enormer Bedeutung. Die Untersuchungen der TLR9 Aktivierung, seiner Signalwege und Auswirkungen auf EBV wurden in EBV-positiven BL Zelllinien durchgeführt. Dabei wurden ebenfalls die TLR Expression in BL Zelllinien und der Einfluß der TLR Aktivierung auf das Zellwachstum und die Zytokin Produktion analysiert.

Diese Arbeit trägt wesentlich zum Verständnis bei, wie EBV und chronische Immunstimulierung, ähnlich wie bei Malaria, einen Einfluß auf die Entwicklung des BL nehmen können. Unsere Daten zeigen, daß eine Aktivierung von TLR9 zu Modifikationen in der Histonstruktur des lytischen EBV Promoters *BZLF1* führt und die lytische Phase unterdrückt wird. Ein Verbleiben von EBV in der latenten Phase erhöht die Wahrscheinlichkeit, dass es zu einer onkogenen Mutation der Zellen kommt. Die hier dargestellten neuen Erkenntnisse dass TLR9 die Aktivierung eines viralen Promoters beeinflussen kann, werden von großer Bedeutung in der Beurteilung der Rolle von TLR9 bei der Tumorentstehung und Entwicklung sein.

Introduction

Epstein-Barr virus

Epstein-Barr virus and its life cycle

The Epstein-Barr virus (EBV) is a double-stranded DNA virus and belongs to the group of the gamma herpesviruses of the *Lymphocryptovirus* (LCV) genus. It infects mainly B-cells and certain epithelial cells and is found in over 90% of the adult human population. Upon primary infection, the host remains a lifelong carrier of EBV. In developing countries, primary infection with EBV usually occurs in early childhood and is often asymptomatic. However, in developed countries, primary infection may be delayed until adolescence or adulthood, resulting in the characteristic clinical features of infectious mononucleosis (IM) in up to 50% of the cases. EBV is transmitted via saliva, and the virus gains access to B-cells within the lymphoid organs in the oropharynx. The virus persists in the host mainly as an asymptomatic latent infection of memory B-cells, which is characterized by only a limited viral gene expression to avoid immune recognition. Occasionally, EBV will switch to its lytic form, which ultimately results in the production of new virus particles, death of the host cell, and infection of new susceptible cells (1, 2). Upon switch to the lytic form, the EBV immediate-early genes *BZLF1* (and its protein Zta) and *BRLF1* (and its protein Rta) are the first viral products expressed. They encode transcription factors that lead to an ordered cascade of early and late lytic gene expression (3).

The physiological stimuli that control the switch between latent and lytic EBV in B-cells are not completely understood. *In vivo*, reactivation of the latent EBV into lytic replication to allow shedding and transmission of the virus probably occurs as memory B-cells differentiate further, e.g., into plasma cells in response to antigen stimulation (3, 4). However, there are several mechanisms known to induce EBV lytic gene expression *in vitro*. These include cross-linking of the B-cell receptor (BCR) with an anti-Ig, treatment with transforming growth factor beta 1 (TGFβ1), addition of Ca^{2+} ionophores or activators of protein kinase C (PKC), like phorbol 12-myristate 13-acetate (PMA) or 12-O-tetradecanoylphorbol-13-acetate (TPA), and infection with human herpesvirus 6 (5-10). All these inducing agents are believed to operate by different mechanisms and different signaling pathways, exemplified by the different duration of stimulus and response time needed for

activation of EBV *BZLF1* and *BRLF1* expression (11). Moreover, these agents exhibit a considerable variability in the degree of activation of lytic EBV infection in established cell lines.

Regulation of the EBV immediate early lytic promoter *BZLF1*

Initiating EBV lytic gene expression by various stimuli involves the activation of the *BZLF1* promoter. The *BZLF1* gene encodes a sequence-specific DNA-binding protein, Zta (also called Z, ZEBRA, or EB1), a member of the bZIP family of leucine-zipper transactivators. The activities of Zta include directly participating in EBV replication via binding to the viral DNA origin of lytic replication, *ori-Lyt*, down-regulating the latency-associated promoters *Cp* and *Wp*, and serving as a transcriptional transactivator of his own promoter, other EBV lytic promoters including the *BRLF1* promoter, *Rp*, and several cellular promoters (3, 12). The *BRLF1* gene encodes a second viral transcription factor, Rta (also called R). Acting together, Zta and Rta play multiple roles in the lytic replication of EBV (3, 12).

Cross-linking of the BCR in Akata cells with anti-human immunoglobulin G (IgG) results in EBV lytic gene expression, starting with the transcription of *BZLF1* (12). Viral gene expression then follows a temporal and sequential order. The immediate-early genes are followed by early and late genes. In EBV virology, these terms are used to describe stages of gene expression in reactivation (12). Viral immediate-early genes like *BZLF1* and *BRLF1* are induced directly by signal transduction from the BCR, independent of the expression of other proteins. *BZLF1* appears to be the major immediate-early protein in EBV (12). Expression of *BZLF1* alone is sufficient to trigger the entire lytic cascade (13, 14). The regulation of the *BZLF1* promoter *Zp* has been investigated extensively. A great amount of data has been collected, still not all regulatory elements within the *BZLF1* promoter have been identified. Most of the regulatory elements seem to be located within the nucleotide (nt) -221 region relative to the promoter's transcription initiation site. In general, in an uninduced resting state, repressive factors bind the *BZLF1* promoter leading to a repressive chromatin structure to keep *Zp* inactive. An important component is MEF-2D, which can associate with histone deacetylases (HDACs) or histone acetyltransferases (HATs) depending on the phosphorylation state of MEF-2D. For instance in Akata Burkitt's lymphoma cells, BCR

signaling activates a signal transduction pathway which can dephosphorylate MEF-2D, reduce its association with HDACs (HDAC4 and HDAC5 of the class II HDACs), and promote its association with HATs (such as CBP or p300) (15, 16). Histone acetylation by HATs is generally associated with open chromatin structure allowing transcription-responsible enzymes to access the promoter. Thus, initial amounts of Zta are generated. The induction of the EBV lytic form requires an initial activation signal of sufficient magnitude to allow expression of enough Zta to feedback, bind and autoactivate its own promoter *Zp*. In addition, Zta activates the adjacent gene *BRLF1*. Together, Zta and Rta activate most of the early genes in the next phase of the lytic infection. This two-step induction model would prevent spurious activation of the lytic form by signals of insufficient magnitude (17).

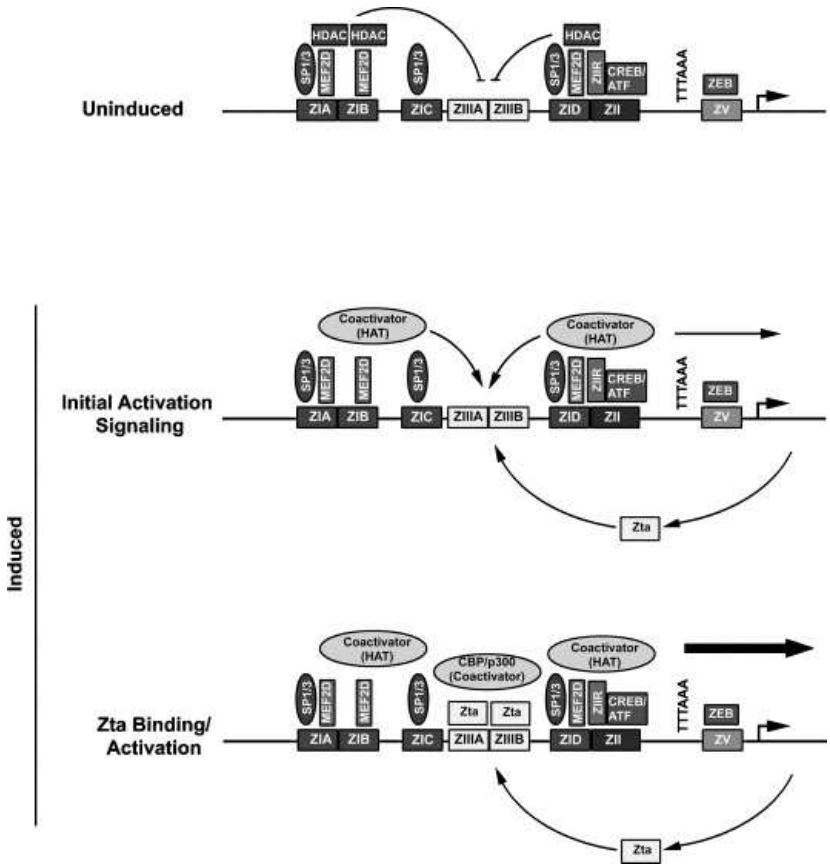

Figure 1. Proposed model for the regulation of *Zp* by Zta (adapted from (18)). Shown are the main regulatory elements located within the nt -221 relative to the promoter's transcription initiation site. In the absence of lytic cycle-inducing signals, suppressor factors such as MEF2D, ZIIR, or ZEB play a role in compacting the local chromatin structure to prevent binding of spuriously expressed Zta or inhibit the activation function of Zta. Induction of the lytic EBV form results in displacement of HDACs and the recruitment of HAT enzymes (co-activators). This leads to initial expression of Zta and opening of the chromatin structure or function. This model predicts that the relaxed chromatin structure allows for the binding or function of newly expressed Zta and a resulting high level of *Zp* activity and commitment to initiation of the lytic form.

Most Zta protein is synthesized from mRNA initiated at *Zp*. The *cis*-acting elements of *Zp* sufficient for both basal and induced activity lie within the nt -221 to +12 region

relative to the promoter's transcription initiation site (see table 1). However, within the *Zp* promoter other (mostly suppressive) regulatory elements upstream the nt -221 to +12 region have been identified. ZEB1 and MEF2D are the only repressors known to bind functionally within the nt -221 to +12 region of *Zp*. Within the *Zp* promoter other repressors that recognize silencing elements have been identified upstream of nt -221 (19, 20). Moreover, the more distal promoter *Rp* can initiate the transcription of a bicistronic mRNA including the *BZLF1* mRNA, and thus lead to expression of Zta (17).

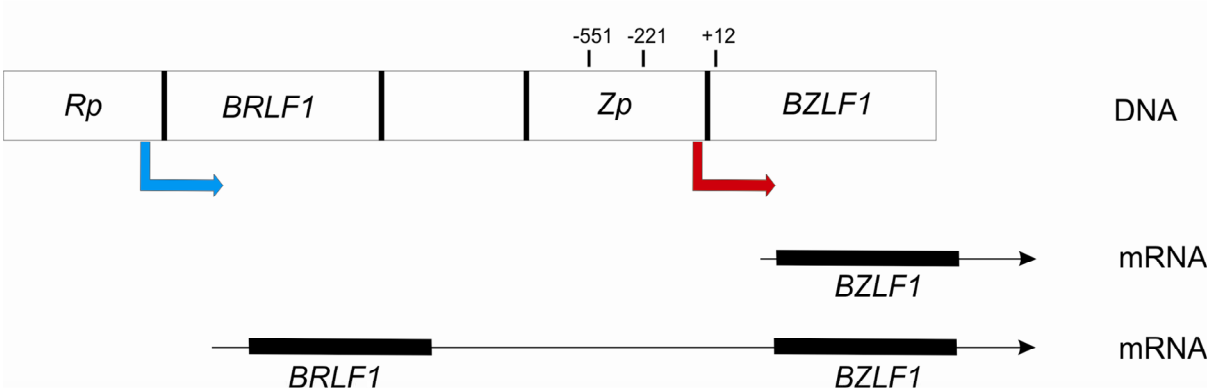

Figure 2. Genomic structure of the responsible promoter regions encoding for Zta (adapted from (21)). Within the nt 221 to +12 region of *Zp* promoter lie the most important regulatory elements for Zta production. Nevertheless other regulator elements upstream nt -221 but within the *Zp* promoter have been identified. Besides transcription from the *Zp* promoter (red arrow), transcription can also start from *Rp* (blue arrow). *Rp* is a promoter region mainly responsible for the expression of the *BRLF1* protein Rta but might also produce Zta due to transcription of a bicistronic mRNA containing *BRLF1* and *BZLF1*.

Table 1. *BZLF1* promoter regions *Zp*, their locations and their functions (adapted from (22)).

Name	Motif	Location	Function
ZI (A-D)	AT-rich	Within -221 to +12 region	can bind the transcription factors Sp1, Sp3, and MEF2D
ZII	CRE-like motif	Within -221 to +12 region	Can bind CREB, ATF family members, C7EBPs, and the AP-1 family of transcription factors
ZIII		Within -221 to +12 region	Contains multiple binding sites for Zta itself
ZIV		Within -551 to -222 region	Transcriptional silencing element
ZV		Within -221 to +12 region	Transcriptional silencing element, binds ZEB1
ZIIR		Within -221 to +12 region	Transcriptional silencing element
HIε		Within -221 to +12 region	Transcriptional silencing element
HIα-HIδ		Within -551 to -222 region	Transcriptional silencing element

In addition to the regulatory elements and the autoactivation model of *Zp*, there is another regulatory mechanism for Zta expression. In anti-immunoglobulin (Ig)-treated Akata cells, Zta, which is produced at the initial stages of induction, may be modified (perhaps through BCR-mediated signaling) in a manner that equips it with a high DNA binding affinity (at least to binding sites within *Zp*). This idea is consistent with the autoactivation model because such a model requires that Zta plays a role in driving its own expression and not necessarily after high levels of Zta have already been produced (18).

EBV-associated malignancies

Burkitt's lymphoma (BL) was first described by the English surgeon Denis Burkitt in 1958 in Uganda as a common cancer affecting children in equatorial Africa (23). The malignancy was found to be dependent on climatic and geographical conditions and this led to the suggestion that a vector-borne virus might be responsible (24, 25). The subsequent discovery of herpesvirus-like particles by electron microscopy in a cell line established from a BL biopsy by Epstein, Achong and Barr in 1964 (26), not only resulted in the initial discovery of EBV but also suggested that EBV is the responsible virus. Later, EBV was found to efficiently transform resting B-cells *in vitro* and induce tumors in nonhuman primates (27-29), confirming the transformation capacity of the virus. The later use of recombinant EBV lacking individual latent genes has confirmed the absolute requirement of EBNA2 an LMP1 in the *in vitro* transformation of B-cells, and has highlighted a critical role for EBNA-LP, EBNA3A and EBNA3C in this process (30). Moreover, EBV is associated with a variety of other human tumors including B-cell malignancies such as Hodgkin's disease (31) and lymphoproliferative diseases arising in immuno-suppressed patients (32), some T-cell lymphomas, and epithelial tumors such as gastric cancer or undifferentiated nasopharyngeal carcinoma (33). All these tumors are characterized by the presence of multiple extrachromosomal copies of the circular EBV genome in the tumor cells and expression of EBV-encoded latent genes, which appear to contribute to the malignant phenotype (30). Different types of tumors are characterized by different patterns of EBV gene expression (see table 2).

Table 2. EBV gene expression in EBV-associated malignancies.

Latency Program	EBNA1	EBNA2-6 EBNA-LP	LMP1, LMP2A, LMP2B	EBER1, EBER2	BARTs	Type of Tumor
I	+	-	-	+	+	Burkitt's lymphoma
II	+	-	+	+	+	Hodgkin's disease, nasopharyngeal carcinoma
III	+	+	+	+	+	Lymphoblastoid cell line, post-transplant lymphoproliferative disease (PTLD), AIDS-associated lymphoma

EBV-associated Burkitt's lymphoma and the contribution of cofactors

Latent EBV is strongly associated with the development of BL, the most common cancer of childhood in equatorial Africa (34). Typical BL is characterized by a chromosomal translocation leading to the deregulation of *c-myc* expression (35), a requirement for cell transformation (36, 37). A key molecular event in BL pathogenesis is the activation of MYC-growth promoting activity (through translocation) and the inactivation of MYC apoptosis-inducing activity (38). Cell lines derived from EBV-positive BL, such as the Akata cell line (39), are instrumental in understanding the factors that regulate the balance between EBV latency and lytic activation. *In vivo*, EBV is thought to contribute to the development of BL by promoting immune evasion, directly transforming lymphocytes, and increasing the chances of a *c-myc* translocation, which can be achieved by increasing the size and survival of the infected B-cell pool (37). In addition, EBV might also have a direct role in tumorigenesis as it might regulate *c-myc* expression, cooperate in the rearrangement of the *c-myc* locus (37, 40, 41), and importantly, might support the inactivation of MYC apoptosis-inducing activity, e.g., through Bim suppression (38). However, the precise role of EBV in the pathogenesis of BL remains to be established, although the detection of monoclonal EBV episomes in EBV-positive BL biopsies suggests that EBV infection preceded proliferation of the precursor B-cells (42). The apparent origin of BL in the germinal centre is based on phenotypic studies and is supported by the ability of BL risk factors such as holoendemic malaria and chronic HIV infection to stimulate proliferation of B-cells in the germinal centre (43). These cells are also programmed to undergo somatic mutation of Ig genes, and this event, in conjunction with the stimulation of germinal centre proliferation and EBV infection,

might be responsible for the generation and selection of B-cells carrying the *c-myc* translocation (30).

The malaria parasite *Plasmodium falciparum* seems to be another crucial factor in the development of BL. Importantly, BL in Africa is restricted to areas where infection with *P. falciparum* malaria is holoendemic. The so-called 'endemic' form of BL occurs at an annual incidence of approximately 5-10 cases per 100,000 children in equatorial Africa and parts of Papua New Guinea. This contrasts sporadic cases of BL worldwide with a 50-fold lower frequency than the endemic form. Several studies indicate that the parasite burden correlates with EBV-positive BL incidence, and that consistent prophylaxis for malaria might lower the risk for EBV-positive BL (44). However, the parasites' contribution to the establishment and survival of the lymphoma is not understood (37, 45), but probably involves multiple immunomodulatory effects and B-cell activation (34, 45). Most noteworthy, data from Parroche *et al.* (46) show that malaria parasite DNA, conjugated to malaria hemozoin, is a ligand for an innate immune receptor named Toll-like receptor 9 (TLR9). This finding becomes significant when one considers that *in vitro* B-cell transformation induced by EBV is efficiently increased in the presence of the TLR9 ligand ODN CpG2006 (47) or *P. falciparum* culture supernatant (48). Malaria parasite DNA might therefore increase EBV-induced transformation *in vivo*. Aside from malaria parasite DNA, a *P. falciparum* membrane protein was recently reported to cause a weak, but detectible, increase in EBV genome copies in Akata cells and human peripheral blood mononuclear cells (PBMCs) by an unknown mechanism (49). *P. falciparum* may thus disturb the balance of latent and lytic EBV by multiple triggers including the parasite membrane protein and TLR9 ligands. The compound effects of these stimuli *in vivo* likely influence EBV pathogenesis and may promote the development of endemic BL. The importance of gaining a clear understanding of how these factors influence EBV-associated lymphomagenesis can not be overstated.

Toll-like receptors and its signaling in B-cells

During my work on EBV and innate immunity, e.g. Toll-like receptors (TLRs), I drafted a review (see below) which we aim to submit in the near future. It covers all the necessary information on TLRs for the investigations described here. Additionally, the draft focuses on the often omitted but extremely important part of TLR9 and BCR interaction. The draft partly includes information about results I generated during the time span of my doctoral thesis and which can be found in the results and attachment section.

Summary

Toll-like receptors (TLRs) are under intensive investigation due to their great potential in pathogen and cancer therapy. As for TLR9, a huge amount of data has been generated concerning its way of function, its signaling and effects. However, most of these studies were performed in mice. As mice and humans comprise great differences in TLR expression, it must be assumed that differences in TLR signaling and function are indeed tremendous. Thus, detailed investigations in the human system are a prerequisite for an accurate understanding of TLRs in pathogen and cancer therapy. Further, most investigations on TLR9 focus on dendritic cells and macrophages, but detailed analyses on TLR9 signaling and its effects in B-cells are often neglected. The scope of this review is to outline human TLR9 signaling and function, with a focus on B-cells including TLR9 – B-cell receptor interaction.

Pattern recognition receptors and Toll-like receptors

Upon encountering a pathogen, the human immune system is equipped with a variety of innate immune receptors in order to eliminate the intruder. This first line of defense is mainly based on the family of the pattern recognition receptors (PRR). They recognize molecules that are broadly shared by pathogens but distinguishable from host molecules. The PRRs include the C-type lectin receptors (CLR) for detecting fungi, the cytosolic RIG-I-like receptors (RLR) and DNA-dependent activator of interferon-regulated factors (DAI) for sensing viral nucleic acid, the nucleotide-binding domain LRR-containing family (NLR) for bacterial products, and the TLRs. In humans, 10 TLRs have been identified so far and ligands

have been established except for TLR10. TLR1-9 recognize specific pathogen-associated molecular patterns (PAMPs) leading to an ordered signal cascade and resulting in gene expression to fight the invading pathogen. The Toll protein, identified previously for its role in dorso-ventral embryo patterning in the fruit fly *Drosophila melanogaster*, was shown to be critical in an anti-fungal response in adult flies in 1996 (50). However, the Toll protein in Drosophila does not directly recognize microbial ligands (51). In 1997, the first human homologue of the Toll protein was identified which is now known as TLR4 (52). TLR4 is also the most studied and to date with the most papers published in the Toll field, in contrast to TLR10 which has very few papers published (according to NCBI PubMed, TLR4 with 4751 papers and TLR10 with 63 papers published until 13th October 2009).

In this review we attempted to incorporate the most contemporary and salient findings in the field of TLR9, including (i) expression profiles and way of receptor function, (ii) activated signaling pathways and effects on gene expression including histone modulation, and (iii) implications on cancer development. Importantly, we aimed to review TLR9 and B-cell receptor (BCR) signaling interaction, as they are crucial for B-cell development as well as for pathogen elimination.

Toll-like receptor expression and signaling

Multiple factors affect the expression patterns of TLRs in the human and mouse system

Human B-cells are characterized by high expression levels of TLR1, 6, 7, 9, and 10 (53-55). However, expression patterns can vary depending on the B-cell subset, the developmental stage of the B-cell, their tissue environment and in B-cell malignancies ((56), (123), and manuscript 3). For instance, naïve human B-cells are barely responsive to TLR stimulation and express only low levels of TLRs (in contrast to mouse naïve B-cells), while memory B-cells are more reactive and more prone to proliferate and differentiate into plasma blasts upon TLR stimulation (53, 54). Further, in the human body the local environment seems to shape the TLR repertoire: TLR9 expression and responsiveness towards the ligand is increased in B-cells isolated from tonsils when compared to those isolated from peripheral blood (57). Importantly, there are distinct differences in TLR expression in mouse cells compared to human cells. Human (non-malignant) B-cells lack TLR4 in contrast to mouse B-

cells. Moreover, in humans only plasmacytoid dendritic cells and B-cells respond to TLR9 ligands whereas in mice B-cells, monocytes, and probably all dendritic cells subsets express TLR9 (58, 59). This differential TLR9 expression pattern in turn affects signaling pathways and results in a unique cytokine and chemokine expression profile. Thus, experimental results from mice cannot easily be extrapolated to humans (51, 60).

Location and signaling of Toll-like receptors

Toll-like receptors can be segregated into two groups due to their cellular location where they recognize their ligands. TLR1, 2, 4, 5, 6 are located on the surface of the cell membrane. Thus, they are designed to recognize structural components of the invading organism, e.g., bacterial cell wall components like proteins, lipoproteins, lipopeptids and lipopolysaccharides. By contrast, TLR 3, 7, 8, 9 are located in the endosomal / lysosomal compartment within the cell. They recognize the less readily accessible nucleotides, e.g., pathogenic RNA and DNA.

In general, TLR engagement by its microbial ligands leads to the interaction with the Toll/IL-R (TIR) domain-containing adaptor proteins, which link activated receptors to downstream kinases that define a given signaling pathway. Depending on which adaptor protein is recruited, different TLRs are able to trigger different signaling pathways. For example, MyD88 and TIRAP function to activate a mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) leading to a pro-inflammatory response. TRIF-related adaptor molecule (TRAM) and TIR domain-containing adaptor protein inducing IFNβ (TRIF) activate TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε) to induce type I IFN production. Together with the expression of costimulatory molecules such as CD40, CD80 and CD86, this leads to the activation of innate immunity and finally to the development of adaptive immunity (61, 62). Moreover, TLRs induce expansion, immunoglobulin class switch recombination and immunoglobulin secretion in human B-cells (63). Interestingly, TLR ligands are known to induce cell-type-specific responses, but how this is achieved is only beginning to reveal. If cell-type specific factors are involved or the subcellular positioning of certain adaptor molecules play a role, is partly unknown (64).

Regulation of TLR9 activation

TLR9 ligands and inhibitors

The DNA-containing unmethylated CpG (cytosine-phosphate-guanine) motif is binding and activating endosomal TLR9. Synthetic oligonucleotides (ODNs) containing the CpG motif are used nowadays to stimulate TLR9-expressing cells in experiments. B-cells can be best stimulated with ODN CpG class B (or K) which have multiple CpG motifs and a phosphorothioate backbone (60). In contrast, dendritic cells are best stimulated with CpG class A (or D) which have mixed phosphodiester-phosphorothioate backbones and contain a single hexameric purine-pyrimidine-CG-puinre-pyrimidine motif flanked by self-complementary bases and induce type I IFNs (65).

In addition to engaging bacterial and viral DNA, TLR9 also recognizes the malaria pathogen *Plasmodium falciparum*, but whether the ligand is hemozoin, a metabolic product of the parasite, or the DNA of the parasite itself is still a matter of debate (46, 66). Endogenous ligands released in the context of tissue damage, cellular stress, or cell death may also be recognized by TLRs. These danger-associated molecular patterns (DAMPS) represent host-derived TLR ligands. These endogenous TLR ligands are involved in solid tumor progression (56). For TLR9, known endogenous ligands are HMGB1 or Ig-DNA complexes. Of note, mammalian self DNA is degraded extracellular by secretion of DNase I into the serum and is therefore not taken up by immune cells for TLR9 engagement.

Besides ligands for TLR9, there are also inhibitors for TLR9 activation known. Inhibitors like chloroquine block TLR9 activation (and TLR3, TLR7, TLR8) by disrupting the acidic milieu in the endosomes (or their maturation) and thus interfere with the activation of all endosomal TLRs (67, 68). This observation has to be evaluated in the context of deactivating proteases and thus interfering in the cleavage of TLR9 to generate a functional receptor. Moreover, short ODNs containing a GGGG motif are able to bind to TLR9, but instead of conformational change and activation of TLR9, the inhibitor blocks TLR9 activation. Thus, they do not interfere with ODN CpG cell binding or internalization (69, 70). Some of these inhibitors are found to act on nanomolar level, although the inhibitors in our hands need to be used in micromolar concentration similar or higher than the concentration for the TLR9 ligands. Stimulatory (CpG), control (GpC) or inhibitory (GGGG) DNA have the similar affinities for TLR9, but only DNA-containing CpG leads to allosteric changes in

the TLR9 cytoplasmic signaling domains and thus activation of TLR9 signaling in the endosomes (71).

TLR9 and its way of function

Toll-like receptors consists of extracellular leucine-rich repeats (LRRs), a transmembrane domain and a cytoplasmic Toll/interleukin-1 receptor homology (TIR) domain. TLR9 initially resides in the endoplasmatic reticulum (ER) of B-cells, dendritic cells and macrophages (in mice), but upon oligonucleotide (ODN) uptake it translocates to the same ODN CpG-containing lysosomal / endosomal compartment for ligand binding and signal transduction (67, 68). Initially, it was thought that TLR9 does not travel through the Golgi but is recruited directly to endo-lysosomes from the ER, bypassing the classical secretory pathway (67). However, recent studies showed that the full-length TLR9 protein is sorted in the ER, traffics through the Golgi and is routed to the endo-lysosome, where it is cleaved by resident proteases (72).

TLR9 is a preformed, inactive homodimer, in which the TIR domains remain separated so that the signaling adaptor, which is MyD88 in the case of TLR9, is not recruited (73). The TLR9 ligand CpG is binding to the endo-lysosomal TLR9 and thus leading to a close apposition of the cytoplasmatic TIR signaling domains (71). These allosteric changes are probably required for the recruitment of MyD88 (71). Moreover, not only receptor binding but also the sequence of potential DNA ligands dictates the quantity and quality of conformational change and subsequent activation of TLR9. Indeed, it is conceivable that ligand secondary and/or tertiary structures as well as binding valency, further influence the receptor response, as noted with the various classes of CpG DNA (71). Of note, other reports have shown that ODN DNA without CpG motifs can be a biologically active ligand for TLR9. Thus, TLR9 recognizes not just CpG motifs, but DNA itself with certain structures (63).

Discrimination of self and non-self TLR9 ligands

The TLR9 ligand consists of a motif found in bacterial and viral DNA. However, most of the approximately 29.000 CpG-rich islands in mammalian cells are unmethylated, which - in theory - make them also suitable to trigger TLR9 (74). Thus, discriminating self and non-self DNA is an important factor in launching immune responses against pathogens

and avoiding immune responses against self-molecules. An interesting study came form Barton *et al.* (75) who described the chimeric TLR9 receptor TLR9N4C that localized to the cell surface and responded normally to synthetic TLR9 ligands but not to viral nucleic acids. This 'relocated' chimeric TLR9 receptor was able to recognize self DNA, which does not stimulate wild-type TLR9. Thus, intracellular localization of TLR9 is required for discriminating between self and nonself nucleic acid – it controls the access of the receptor to different sources of DNA - but not for ligand recognition (75). Moreover, there is a requirement for capsid degradation by lysosomal proteases so that nucleic acids of the viruses become accessible for endosomal TLR9. Purified viral DNA is a poor inducer of TLR9 compared with intact virus.

Another strategy for TLR9 to prevent responding to self nucleic acids involves cleavage of TLR9 to create a functional receptor. The ectodomains of TLR9 (and TLR7) are cleaved in the endo-lysosome, such that no full-length protein is detectable in the compartment where ligand is recognized. Although both the full-length and cleaved forms of TLR9 are capable of binding ligand, only the processed form recruits MyD88 on activation, indicating that this truncated receptor, rather than the full-length form is functional (72). The ectodomain cleavage represents a strategy to restrict receptor activation to endo-lysosomal compartments and prevent TLRs from responding to self nucleic acids. TLR9 receptors found on the cell surface may be prevented from responding inappropriately to self nucleic acid ligands (72). Thus, a low pH seems not to be necessary for TLR9 activation by CpG DNA, as the chimeric TLR9N4C responded to CpG DNA in the presence of chloroquine or bafilomycin A1, but might be necessary for the proteases-induced TLR9 cleavage to generate the functional short receptor form.

Toll-like receptor 9, its signaling pathways and effects in human B-cells

Sequence-specific recognition of CpG DNA is believed to occur in the endo-lysosomes after non-specific DNA uptake into the cells. The uptake into lymphocytes is energy and temperature dependent and greatly increased by cell activation (60). Moreover, it was suggested that different classes of Phosphatidylinositol 3-kinases (PI3K) are involved in uptake / vesicular trafficking of CpG DNA to the endosomal compartment, whereas other PI3K classes might participate as a signaling component as well (63, 76). For example, PI3K

delta functions in influencing the type of B-cell cytokine production and differentiation response induced by TLR4 and TLR9 ligands (77). Importantly, PI3K is known to activate the AKT (PKB) pathway. During an infection, endocytosis of microbial nucleic acids, which may occur upon bacterial disintegration or cellular infection via cell-surface receptor-mediated uptake, is necessary (56). Nevertheless, after ligand binding in the endosomes, TLR9 is induced to conformational changes and thus recruits the myeloid differentiation marker 88 (MyD88). Signaling of TLR9 in B-cells proceeds only through MyD88 (73), which then activates the IL-1R-associated kinase 1 (IRAK1), the TNF-receptor-associated factor 6 (TRAF6), and the TGFβ-activated kinase 1 (TAK1) pathway (78, 79). TAK1 phosphorylation leads to the activation of the nuclear factor-κB (NF-κB) transcription factor (see Figure 3 on page 20). TAK1 also signals through mitogen-activated protein kinases (MAPKs), such as p38 and the c-Jun NH_2-terminal kinase (JNK), leading to the activation of activating protein-1 (AP-1) complexes (78). Thus, NF-κB and AP-1 complexes are able to translocate into the cell nucleus where they promote the expression of genes involved in B-cell activation, proliferation and production of inflammatory cytokines as a part of the immune response against pathogens (79). Moreover, activation of CCAAT/enhancer binding protein (C/EBP), cAMP-responsive element-binding protein (CREB) and the Ras-ERK pathway after TLR9 engagement could also be observed (56, 79). Another class of transcription factors, the family of interferon-regulated factors (IRFs, e.g. IRF3, IRF5, IRF7) is activated after TLR engagement, interacts with MyD88 and translocates to the nucleus where they promote the expression of type I interferons (of note, IRF5 induces expression of proinflammatory cytokines). However, reports indicate that TLR9-induced ERKs and IRFs play less prominent roles in B-cells than in (mice) macrophages or dendritic cells where they promote IL10 and type I IFN expression, respectively (79, 80).

Although human B-cells are generally considered poor cytokine producers, stimulation with TLR7 or TLR9 ligands results in the secretion of pro-inflammatory cytokines like IL-1β, IL-2, IL-6, IL-8, and to the release of immune regulatory cytokines that might limit the intensity of the inflammatory response, such as IL10 (56). Further, TLR7/9 stimulation in naïve and transitional human B-cells induces differentiation in the absence of significant proliferation in these B-cell subsets. A proliferative response to TLR7/9 ligands is predominantly observed in IgM+ memory B-cells (123). Moreover, TLR triggering of terminally differentiated plasma cells augments Ig production (see manuscript 3). Finally, TLR9 plays a role in the induction of T helper 1 (Th1) acquired immune response (63).

The differential activity of the ODN different classes of the TLR9 ligands on immune cells and how the signals are transduced can not be explained fully to date. Although it is known that different IRFs are activated depending on the type of CpG used, we do not know how the different effects and activated proteins are produced from the same TLR. An interesting study came from Guiducci *et al.* who demonstrated in plasmacytoid dendritic cells that the differential higher order structure and the endosomal location of the ODN classes determines the biological response (IFNα production versus maturation) of the cells towards TLR9 ligands (81). Moreover, additional factors / receptors might also be involved in shaping the diverse signaling and outcome of TLR9 engagement (65).

Signaling interaction of Toll-like receptor 9 and the B-cell receptor (BCR)

The BCR in immature and mature B-cells

Besides germ line-encoded PPRs like the TLRs, B-cells express clonally rearranged antigen (Ag)-specific receptors on their surface. The BCR has two main roles. The first is to transmit signals that regulate B-cell fate decisions. The second is to mediate antigen processing, leading to the presentation of antigen to T-cells, which allow full activation of B-cells in the effector phase.

In pre-B-cells, rearrangements of the Ig light chain are completed. An important role of the BCR at the later immature B-cell stage is to induce efficient elimination of the potentially harmful, self-reactive B-cells, which can be achieved in three ways: immature B-cells are eliminated through negative selection (BCR-induced cell death), they are inactivated (anergy), or they revise the specificity of their BCRs (receptor editing) (82). Immature B-cells that have passed successfully through developmental checkpoints in the bone marrow emigrate subsequently to the spleen as transitional B-cells, which then become mature B-cells. Mature B-cells are classified into subsets, e.g., follicular B-cells or marginal-zone B-cells. Depending on the developmental stage, the BCR mediates distinct cell-fate decisions at the immature and mature stages of B-cell development leading to receptor editing/cell death or activation/differentiation, respectively. Moreover, the physical form and/or the context of the Ag, the availability of T-cell help, different expression and activation levels of kinases, phosphatases and adaptor molecules, and involvement of co-receptors will contribute to the

diverse outcome of the BCR triggering (82). It might seem reasonable to expect signaling via BCR engagement on immature B-cells to differ from mature B-cells (83). Indeed, a number of variations in the signaling cascade of immature and mature B-cells have been identified and invoked to explain differences in cellular outcomes. Gene chip analysis has also revealed increases in distal signaling molecules (NF-κB, JAKs, MAP kinases), anti-apoptotic molecules (Bcl-2, A1), and inhibitory co-receptors (CD22, FcγRIIB) expression as cells mature (84). Of note, BCR translocation of lipid rafts is non-functional in immature primary B-cells and immature B-cell lines compared with mature B-cells (85, 86). Differential recruitment to rafts might result in qualitative differences in downstream signaling profiles. Instead of inducing proliferation or differentiation, immature B-cells respond to BCR cross-linking by increasing expression of genes involved in receptor editing and/or apoptosis (87, 88). For instance, protein kinase C (PKC), which prevents BCR-induced apoptosis of mature B-cells is impaired selectively in BCR-activated primary immature B-cells (89, 90).

BCR-induced signaling pathways

If the BCR is engaged by an antigen, BCRs will form signaling active microclusters, followed by a conformational change to an 'open form' (44, 91). The first signaling events consist of recruitment of Lyn and Syk (which phosphorylates the cytoplasmatic signaling domain of the BCR) to the BCR microcluster, and Ca^{2+} release. The four major signaling pathways activated include phospholipase C (PLC), the Rho family of GTPases like VAV1-3, Ras, and phophatidylinositol-3-kinase (PI3K) (92, 93). They lead to the activation of MAP kinase signaling cascades and of transcription factors like NF-κB, and thus to transcription of a variety of genes depending on the maturation state of the cell (see Figure 3 on page 20). The activated MAP kinases - consisting of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminas kinase (JNK/SAPK) and p38 MAPK - phosphorylate different sets of transcriptions factors including Elk1 and c-Myc by ERK, c-Jun and ATF2 by JNK, and ATF2 and MAX by p38 MAPK. Studies have shown both positive (survival/proliferation) and negative (apoptosis/deletion) roles for ERK depending on the maturational state and activation status of the B-cell (90). In addition, the BCR is internalized and thus efficiently transports antigens to a MHC-class II-containing, multivesicular, intracellular compartment in which the antigens are proteolytically cleaved and the resulting peptides are assembled with MHC class II molecules into complexes for recognition by helper T-cells (94).

BCR and its interaction with co-receptors

BCR signaling is regulated and fine tuned by several co-receptors. These include the B-cell inhibitory receptor FcγRIIb (recognizes immune complexes) and the stimulatory co-receptor complex CD19/CD21 (recognizes complement-coupled antigens). However, the family of the TLRs seems to be a new player in the field. During an infection, B-cells will most probably receive signals through both TLRs and BCRs. Investigations how these receptors and their signaling interact are crucial for the understanding of the human immune system towards a pathogen and, importantly, autoimmune diseases like systemic lupus erythematodes. The synergistic engagement of TLR9 and BCR in response to DNA-containing antigens has been implicated in the activation of autoimmune B-cells (95). Moreover, TLR9 has been shown to play a critical role in regulating DNA-specific autoantibody production in mouse models of lupus by mechanisms that involve the simultaneous engagement of TLR9 and the BCR (96).

The diverse outcome of BCR – TLR9 interactions is dependent on the maturation state of the B-cell

TLR9 signaling has the ability to interfere with BCR signaling, leading to synergistic or blocking effect depending on the maturation step of the B-cell (97). In immature, B-cells, TLR9 and BCR synergize with cytokines production (like IL10, IL6, TNFα), but TLR9 blocks BCR mediated growth arrest and apoptosis (by interfering with the regulation of *c-myc* and *bcl-xl*) (98-100). In mature B-cells, TLR9 and BCR synergize in B-cell proliferation and production of cytokines and Ig production (101, 102).

Little is known on the molecular events integrating TLR signaling into the classical BCR-mediated signaling cascades. Only a few studies attempted to distinguish BCR- from TLR signaling. TLR9 engagement seems not to interfere with the early signaling pathway induced by surface Ig cross-linking ((103) and manuscript 1), but may work through an intracellular pathway at a later stage. As with the BCR, the TLR9-initiated signaling pathways ultimately result in phosphorylation of MAP kinases, activation of PI3K, and activation of transcription factors like NF-κB and AP1. However, these TLR9–initiated signaling pathways are partly distinct from those triggered by the BCR (92, 93, 104). Indeed, BCR and TLR9 co-stimulation induced synergistic activation of the MAP kinase p38 and JNK for cytokine production in immature B-cells, but ERK is not enhanced (97). This is in

contrast to the synergistic activation of ERK in addition to p38, JNK for the induction of mature B-cell proliferation. Moreover, TLR9 and BCR engagement seem to activate different sets of MAPK and transcription factors via distinct signaling pathways. BCR-mediated signals strongly activate MEK1/2, MKK3, MKK6 and its downstream targets ERK and p38 (but have little effect on MKK4, and JNK). Moreover, MAP kinases have differential regulatory roles for CpG-mediated cytokine versus cell proliferation depending on the maturity of the B-cells (60). In summary, although interaction of signaling pathways of TLR9 and BCR at the level of NF-κB and MAPKs have been observed, one has to consider that different sets of MAPKs and NF-κB subunits are involved which might explain the diverse outcome depending on the maturation state of the B-cell. Activation of ERK is important in BCR signaling but less important in TLR9 signaling in B-cells; and JNK has a less prominent role in BCR triggering than in TLR9 activation. However, the relatively few studies concerning the signaling interaction of BCR and TLR9 limit the validity of the herein stated conclusions.

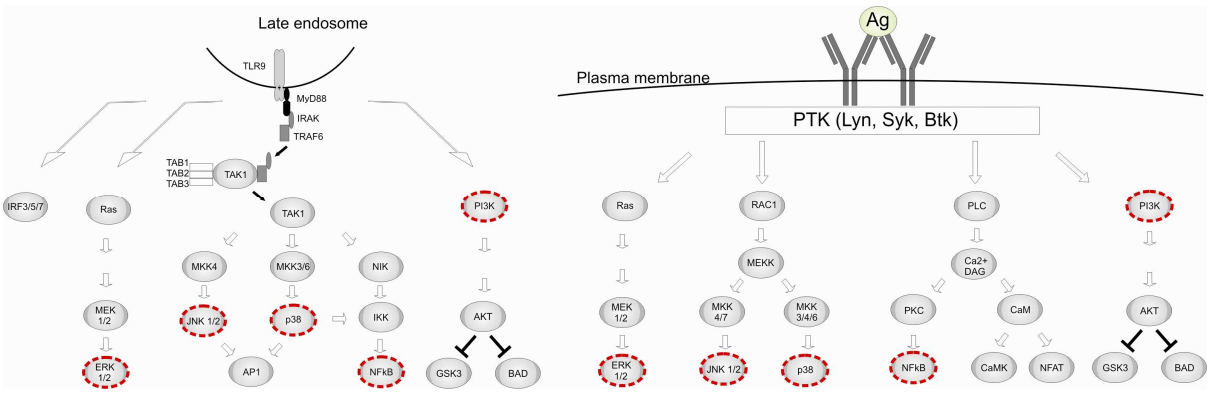

Figure 3. TLR9- and BCR-induced signaling cascades. The main important signaling pathways are shown. Cross-talk between signaling components of the single TLR9 or BCR pathway exists: The PLC-Ca^{2+}/PKC axis can induce MAP Kinases (JNK, p38, ERK); DAG cross-talks to RAS-RAF-ERK; PI3K/AKT cross-talks to IKK-NF-κB; the PI3K can induce Ras activation. Possible interactions between TLR9 and BCR signaling might take place at the MAPK and NF-κB level (highlighted by red dashed lines), but also downstream of the MAPKs in the nucleus like CREB or ATF-2. Of note, the TLR9-induced signaling pathway to IRFs and Ras-ERK1/2 seems to be less prominent in B-cells, but important in pDCs and mouse macrophages. However, activation of ERK is important in BCR signaling. JNK has a less prominent role in BCR triggering than in TLR9 activation. Abbreviations are as followed: CaMK, calcium-modulin-dependent kinase; ERK, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase 3; IKK, inhibitor of NF-κB kinase; JNK, c-JUN NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK/ERK kinase 1/2; MEKK, MAPK/ERK kinase kinase; MKK 3/4/6/7, MAPK kinase 3/4/6/7; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; PI3K, Phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinases.

TLR9 and BCRs meet at the autophagosomes in mature B-cells

The next obvious question is how signaling interaction between TLR9 and BCR can occur as TLR9 and the BCR initiate signaling from two spatially distinct sites, the TLR9 from endosomes, and the BCR from the plasma membrane. A study from Chaturvedi *et al.* (105) demonstrated that upon antigen binding, the BCR in mouse B-cells initiates signaling at the plasma membrane and continues to signal to activate MAP kinases as it traffics to autophagosome-like compartments. Further, the internalized BCR signals to recruit TLR9-containing endosomes to the BCR-containing autophagosome where hyperactivation of MAP kinases occurs. Thus, TLR9 and BCR signaling appears to synergize in autophagosome-like structures and hyperactivate signaling through to p38 and JNK (105). Of note, engagement of BCR and TLR9 can be achieved by individual triggering of the receptors, or by CpG linked to protein antigen. Recently it was demonstrated that CpG linked to protein antigen gain selective entry into Ag-specific B-cells through BCR-mediated endocytosis, allowing engagement of intracellular TLR9, and resulting in enhanced B-cell proliferation and production of antigen-specific class-switch antibodies *in vivo* (106). The individual triggering of BCR and TLR versus triggering of the receptors with CpG linked to protein antigen might not only yield in differential activated signaling pathways but also in effector function. In the case of TLR4, studies in mouse B-cells showed that simultaneous stimulation of TLR4 and BCR additively elevates B-cell activation, but co-engagement of TLR4 and BCR by antigen-coupled LPS synergistically enhances activation of B-cells (107). Thus, depending on the nature of the trigger, there can be different outcomes due to differences in activation of signaling pathways.

The BCR in the context of TLR Tolerance

Another interesting aspect of signaling interaction between TLR9 and BCR was observed recently and involves TLR tolerance. TLR tolerance is defined as a temporary hypo-responsiveness of immune cells to TLR ligands following repeated or chronic stimulation through the same or different TLRs. (108). For instance, pre-stimulation of TLR7 reduces the response of a subsequent stimulation of TLR7 or TLR9 in human and mouse cells. Importantly, triggering the BCR rescues B-cells from TLR tolerance (109). Of note, TLR tolerance is not affecting all TLR-mediated functions. This might be partly explained by a recent study, which proposed that repeated TLR stimulation and its effect on selected but

not all TLR-responsive promoters might be regulated at the level of histone modifications of individual promoters (110), which will be discussed later in the text.

Expression interference of BCR and TLR9

As TLR9 and BCR can interfere with each others signaling, they can also interfere with each others expression. Human naïve B-cells express most TLRs at low to undetectable levels, but expression of TLR9 and 10 is rapidly induced following BCR triggering (54). Memory B-cells express several TLRs at constitutively high levels. The differential expression of TLR9 correlates with its responsiveness to its agonist, CpG. Thus, human memory B-cells proliferate and differentiate to Ig secreting cells in response to CpG, while naïve cells only do so if simultaneously triggered through the BCR.

Toll-like receptor 9, regulation of gene expression and histone modifications

Transcription factors like NF-κB, AP1, IRFs and others are crucially important to transduce the signal from the TLR9 to the promoters and thus leading to changes in gene expression. The regulation of NF-κB activation and other transcription factors have been intensively reviewed and will not be summarized here.

In addition to activation of transcription factors, host gene expression can be initiated or modified by the architecture of the chromatin, e.g., DNA or histone modifications. Nuclear DNA is packed into nucleosomes, which consist of a histone octamer core around which DNA (approximately 143bp) is wrapped. The core histones are reversibly modified by acetylation, methylation, ubiquitination, biotinylation and phosphorylation. Modifications occur on the N- and C-terminal tails of the core histones, and more recently analyses of histone modifications by mass spectrometry have revealed several modifications (acetylation and methylation) in the histone fold (111). Among the N-terminal histone tail modifications, acetylation is perhaps the most characterized and has been found associated with actively transcribed regions of chromatin (111). Histone acetylation is the covalent addition of acetyl groups on lysine residues of histones and non-histone proteins that renders chromatin accessible to DNA binding proteins.

TLR signaling results in the general activation of transcription factors such as AP-1 and NF-κB. Histone regulation might be a possibility of how gene expression due to TLR-mediated transcription factor activation can be specifically modified to adapt to the invading organism or to avoid excessive inflammation.

TLR-induced chromatin remodeling on the IL12p40 promoter has been described, indicating that chromatin remodeling is an additional level of TLR signaling specificity (112, 113). Recently, Foster *et al* found out that individual host gene promoters can be targeted and modulated in its histone architecture after TLR engagement (110). Repeated triggering of TLRs was shown to repress activation of selected TLR-responsive promoters (TLR tolerance), while other TLR-responsive promoters were not affected (110). This adaptation of gene expression includes host genes promoters from cytokines, but TLRs also directly interfere with histone structure of pathogen gene promoters. Recently, our group provided strong evidence that TLR9 is changing the acetylation and phosphorylation states of histones on the B-cell tropic Epstein-Barr virus (EBV) lytic gene promoter *BZLF1* leading to a suppression in lytic gene expression (see manuscript 1). Thus, TLRs can interfere with lytic activation of a chronic pathogen like EBV residing in its latent form via alteration of histones on the viral gene promoter.

However, no study has identified the exact mechanism by which histone remodeling occurs. It can only be speculated how TLR9 induces changes in the histone architecture. TLR engagement strongly induces distinct sets of MAP kinase kinases (MKKs/MEKs) as well as MAPK phosphatases, e.g. DUSP1 (MAPK phosphatase 1) (114). In turn, these enzymes are able to differentially regulate the activity of the AGC kinase family members, e.g., MSK1/2 and RSK2, which are known to affect histone phosphorylation (114, 115). In the context of histone acetylation, TLR9 might affect the recruitment of co-activators which have histone deacetylase (HDAC) or histone acetyl-transferase (HAT) activities (like p300/CBP), leading to chromatin remodeling on host and viral promoters and subsequent changes in gene expression. Importantly, MSKs, HDACs and HATs are known to modify a range of other non-histone proteins including transcription factors such as CREB, ATF-1, and NF-κB. Thus, detailed investigations about their effect on transcription factor activation versus histone modification are needed.

Toll-like receptor 9: friend or foe in cancer development and treatment

Carcinogenesis is a progression of events resulting from alterations in the processing of the genetic information. These alterations result from stable genetic changes (mutations) and potentially reversible epigenetic changes, including DNA methylation and histone modifications (111). The clinical interest for TLRs in cancer research is increasingly growing. Among TLR ligands those engaging TLR9 are the most intensively studied as they can be relatively simply manufactured, administrated through any drug route e.g., intramuscular, subcutaneous or orally, and have very low side but very strong adjuvant effects that polarize helper T-cell responses to Th1 (63, 79). Th1 immune activation is optimized for fighting intracellular infections such as viruses and involves the activation of natural killer (NK) cells and cytotoxic T-lymphocytes (CTLs) that can lyse infected cells. This type of immune activation is the most highly desired for cancer therapy, as the same defenses (activation of NK cells, monocytes and macrophages and induction of cytokines with direct or indirect antitumor activities) can be directed to kill tumor cells (60). TLR activation might enhance cancer immunotherapy by activation of tumor-specific CTLs which in principle can eradicate even metastatic tumor cells. Thus, for the TLR9 agonist ODN CpG there are several phase II and phase III clinical trials as adjuvants to cancer vaccines and in combination with conventional chemotherapy and other therapies being tested (60).

Having a great potential in cancer therapy, TLR stimulation due to excessive pathogen encounter might lead to a high inflammation process over a long period. As inflammation is one of the reasons for a cell to become malignant, TLR activation might not only be beneficial but detrimental for humans (116). In humans, chronic active infection and inflammation are considered two of the most important epigenetic and environmental factors contributing to tumorigenesis and tumor progression (117, 118). Tumor environment contains significant amount of immune infiltrates and has all the characteristics of an ongoing inflammatory process (119). Moreover, when a tumor is already established, TLRs might induce tumor promoting factors and tumor immune evasion; for example, IL12 induces resistance of tumor cells to CTL and NK cell attack and evasion from immune surveillance and matrix metalloproteinase promotes cell invasion. The transcription factor NF-κB upregulates the expression of many pro-inflammatory cytokines, chemokines, growth factors, matrix metalloproteinases, adhesion molecules and, more importantly, anti-apoptotic proteins like c-FLIP and XIAP, and is able to inhibit pro-apoptotic proteins such as Bax and Caspase

9 (120). These cellular events may lead to chemo-resistance (119). Moreover, in cancer cells, certain genes required for development or for apoptosis are silenced in the transformation process of the cells, leading to uncontrolled proliferation. The change of gene expression partly relies on enzymes like histone-deacetylases (HDACs), inducing chromatin remodeling processes and leading to differentially expressed genes (121). That histone modification can induce proliferation of cells to cause leukemia is well known in the case of thyroid hormone receptor. As TLRs are known to induce histone modification, TLRs might be beneficial or detrimental in tumor development and has to be evaluated for the specific case. Inhibiting certain TLRs in inflammation-associated cancers might therefore yield new therapies (121). The use of histone-deacetylases (HDAC) inhibitors might therefore counteract the detrimental triggering of TLRs. Our group has shown that TSA could reverse the effect of TLR9 on human and viral gene expression (see manuscript 1). That HDAC inhibitors – beside our findings – have great potential in cancer therapy is widely accepted. Although both normal and tumor cells become enriched in acetylated histones, sensitivity to HDAC inhibitors is ten-fold higher in tumor cells (111).

Toll-like receptor 9: future directions

Although TLRs seem to have great potential in cancer therapies due to their ability of raising or enhancing immune responses against malignant cells, TLRs might as well play a supportive role in tumor development and progression via expression of tumor promoting factors regulating apoptosis. Indeed, chronic active infection and inflammation are assumed to stimulate B-cell tumor development. A model from Chiron and colleagues (56) proposed that repeated polyclonal activation of leukemic B-cells by microbial molecules during natural infection or inflammation is the initial step in the oncogenic process that lowers the threshold for outgrowth of malignant cell clones. Evaluating if TLR9 has a positive or negative effect in tumor onset and progression has to be done on the cell type, maturation of the cell, whether an infection is present, and other factors separately. Investigation on TLR9-activated signaling pathways – sub-pathways, strength and duration of activation – may give important information whether TLRs might have beneficial or detrimental effects on tumor development. In this matter, TLR triggering gives a ‘signaling fingerprint’, which has to be

carefully examined as it might contain the key for the understanding of the diverse outcome of TLR engagement.

Subject of Investigation

As my work deals with TLRs and EBV gene expression, the following topics were subjected for investigation:

1) TLR9 triggering induces suppression of EBV lytic gene expression via histone modification (manuscript 1)

In this (first authorship) paper I characterized and revealed the mechanism of how TLR9 triggering suppresses EBV lytic gene expression in Akata BL cells. Moreover, TLR9 was found to suppress EBV in Mutu BL cells and MHV-68 in S11 cells, indicating a general effect of TLR9 on gamma herpesviruses from different species and in distinct cellular backgrounds. This manuscript is the main part of my work and thus will be the essential part in the discussion and outlook section.

2) Immune activation suppresses initiation of lytic Epstein-Barr virus infection (manuscript 2)

The paper describes the initial discovery that triggering the innate immune system affects EBV gene expression. My contribution to this crucial finding was that, in the Akata cell line, TLR9 suppresses BCR-induced EBV lytic gene expression. Thus, I established the Akata cell line as a model system for the TLR9-induced suppression of EBV, which was used throughout the following investigations.

3) Plasma cell Toll-like receptor (TLR) expression differs from that of B-cells and plasma cell TLR triggering enhances immunoglobulin production (manuscript 3)

As the differential expression of the TLRs correlates with its responsiveness to its agonists, we investigated the expression patterns of TLRs in primary B-cells and B-cell lines. Using quantitative real-time PCR we measured mRNA expression of the Toll-like receptors in B-cell lines (not shown in this publication) and primary B-cells. This helped to shed light on the different expression patterns in B-cells depending on the developmental stage and the tissue environment, and showed for the first time that triggering of plasma cell TLRs enhances immunoglobulin production.

4) Latent membrane protein 2B regulates susceptibility to induction of lytic Epstein-Barr virus infection (manuscript 4)

Inducing EBV lytic gene expression in the model cell line Akata starts with B-cell receptor cross-linking. My project involved identifying the first steps of BCR signaling and whether they are affected upon TLR9 triggering. Therefore, I designed experiments for investigating the first steps of BCR signaling including Ca^{2+} flux measurements which I also performed in this publication. Thus, we could show EBV's LMP2B counteracting LMP2A's function by restoring BCR-induced calcium mobilization. Our findings suggest that LMP2B negatively regulates the function of LMP2A in preventing the switch from latent to lytic EBV replication.

5) Burkitt's lymphoma cell lines as a model B-cell system for investigating TLR engagement and interaction with the BCR

Here, I investigated the outcome of TLR triggering in BL cell lines in the context of proliferation and cytokine expression. Importantly, effects of TLR9 and BCR signaling interaction were studied using microarray and quantitative PCR analysis to identify overlapping and segregated gene expression profiles for BCR and TLR9 engagement. This (first-authorship) manuscript is in preparation.

Results

TLR9 triggering induces suppression of EBV lytic gene expression via histone modification

Ludwig Zauner, Gregory T. Melroe, Markus P. Rechsteiner, Marcus Dorner, Martina Arnold, Christoph Berger, Michele Bernasconi, Beat W. Schaefer, Roberto F. Speck, David Nadal

Manuscript submitted for publication.

Abstract

By recognizing numerous pathogen-associated DNA sequences, Toll-like receptor 9 (TLR9) causes a strong innate immune response resulting in cytokine secretion and costimulatory molecule expression. Immune activation with a TLR9 ligand was shown to suppress the initiation of EBV lytic replication in primary human B-cells. Here, we show that the suppression of EBV lytic gene expression is dependent on functional TLR9 and MyD88 signaling in the Akata cell line. Furthermore, TLR9 activation suppresses not only human but also murine gamma herpesvirus lytic gene expression, independently of the pathway involved in reactivating the virus. Finally, suppression of EBV lytic gene expression due to TLR9 activation in Akata cells involves histone modifications impacting on the activation of a viral lytic gene promoter. The suppression of EBV lytic gene expression, thus EBV hiding in latency, via histone modifications may have evolved to protect the virus against a TLR9-induced immune response.

For detailed information see attached manuscript 1. This manuscript is the main part of my work and thus will be the essential part in the discussion and outlook section.

Immune activation suppresses initiation of lytic Epstein-Barr virus infection

Kristin Ladell, Marcus Dorner, Ludwig Zauner, Christoph Berger, Franziska Zucol, Michele Bernasconi, Felix K. Niggli, Roberto F. Speck and David Nadal1 (2007) Cell Microbiol., Aug;9(8):2055-69.

Abstract

Primary infection with Epstein-Barr virus (EBV) is asymptomatic in children with immature immune systems but may manifest as infectious mononucleosis, a vigorous immune activation, in adolescents or adults with mature immune systems. Infectious mononucleosis and chronic immune activation are linked to increased risk for EBV-associated lymphoma. Here we show that EBV initiates progressive lytic infection by expression of *BZLF1* and the late lytic genes gp85 and gp350/220 in cord blood mononuclear cells (CBMC) but not in peripheral blood mononuclear cells (PBMC) from EBV-naive adults after EBV infection ex vivo. Lower levels of proinflammatory cytokines in CBMC, used to model a state of minimal immune activation and immature immunity, than in PBMC were associated with lytic EBV infection. Triggering the innate immunity specifically via Toll-like receptor-9 in B-cells substantially suppressed *BZLF1* mRNA expression in acute EBV infection ex vivo and in anti-IgG-stimulated chronically latently EBV-infected Akata Burkitt lymphoma cells. This was mediated in part by IL12 and IFNγ. These results identify immune activation as critical factor for the suppression of initiation of lytic EBV infection. We hypothesize that immune activation contributes to EBV-associated lymphomagenesis by suppressing lytic EBV and in turn promotes latent EBV with transformation potential.

For detailed information see attached manuscript 2.

Plasma cell Toll-like receptor (TLR) expression differs from that of B-cells and plasma cell TLR triggering enhances immunoglobulin production

Marcus Dorner, Simone Brandt, Marianne Tinguely, Franziska Zucol, Jean-Pierre Bourquin, Ludwig Zauner, Christoph Berger, Michele Bernasconi, Roberto F. Speck, and David Nadal;
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Abstract

Toll-like receptors (TLRs) are key receptors of the innate immune system and show cell-subset specific expression. We investigated the mRNA expression of *TLR* genes in human haematopoietic stem cells (HSC), naïve B-cells, memory B-cells and plasma cells from palatine tonsils and plasma cells from peripheral blood. HSC and plasma cells showed unrestricted expression of *TLR1-TLR9*, contrasting B-cells which lacked *TLR3*, *TLR4* and *TLR8* but expressed mRNA of all other *TLR*s. We demonstrate for the first time that TLR triggering of terminally differentiated plasma cells augments immunoglobulin production. Thus, boosting immediate antibody response by plasma cells upon pathogen recognition may point to a novel role of TLRs.

For detailed information see attached manuscript 3.

Latent membrane protein 2B regulates susceptibility to induction of lytic Epstein-Barr virus infection

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Abstract

The B-lymphotropic Epstein-Barr virus (EBV) encodes two isoforms of latent membrane protein 2 (LMP2), LMP2A and LMP2B, which are expressed during latency in B-cells. The function of LMP2B is largely unknown, whereas LMP2A blocks B-cell receptor (BCR) signaling transduction and induction of lytic EBV infection, thereby promoting B-cell survival. Transfection experiments on LMP2B in EBV-negative B-cells and the silencing of LMP2B in EBV-harboring Burkitt's lymphoma-derived Akata cells suggest that LMP2B interferes with the function of LMP2A, but the role of LMP2B in the presence of functional EBV has not been established. Here, LMP2B, LMP2A, or both were overexpressed in EBV-harboring Akata cells to study the function of LMP2B. The overexpression of LMP2B increased the magnitude of EBV switching from its latent to its lytic form upon BCR cross-linking, as indicated by a more-enhanced upregulation and expression of EBV lytic genes and significantly increased production of transforming EBV compared to Akata vector control cells or LMP2A-overexpressing cells. Moreover, LMP2B lowered the degree of BCR cross-linking required to induce lytic EBV infection. Finally, LMP2B colocalized with LMP2A as demonstrated by immunoprecipitation and immunofluorescence and restored calcium mobilization upon BCR cross-linking, a signaling process inhibited by LMP2A. Thus, our findings suggest that LMP2B negatively regulates the function of LMP2A in preventing the switch from latent to lytic EBV replication.

For detailed information see attached manuscript 4.

Burkitt's lymphoma cell lines as a model B-cell system for investigating TLR engagement and interaction with the BCR

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Abstract

During an infection, B-cells will most probably receive signals through both TLRs and BCRs. Investigations of how these receptors of innate and adaptive immunity and their signaling interact, are crucial for the understanding of the human immune system towards a pathogen. Burkitt's lymphoma cell lines are used as a model to study the switch of EBV latent to EBV lytic gene expression. As TLR9 was reported to suppress BCR-induced EBV lytic gene expression, we aimed to investigate in more detail the effects of TLR9 on host gene expression and proliferation. Importantly, we focused on the effects of TLR9 and BCR triggering to shed light on the signaling interactions between these two receptors. We could show that TLR9 induces cytokine expression in BL cells peaking in the sequential order IL8 / TNFα, followed by IFNγ and IL10. Interestingly TLR9 engagement does not affect proliferation rates of Akata cells. Moreover, BCR triggering results in either a synergistic or suppressive effect on TLR9-induced cytokine production depending on the investigated cytokine. Finally, microarray analysis was performed to identify overlapping and segregated gene expression profiles for BCR and TLR9 engagement in the BL cell line Akata.

Discussion and Outlook

Burkitt's lymphoma cell lines are essential for investigating the switch from EBV latent to lytic gene expression. As the EBV-mediated transformation of B-cells takes place during EBV latency, it is crucial to study the regulatory mechanisms of latent EBV versus lytic EBV gene expression. Moreover, as lytic gene expression results in the death of the host cell, the switch to lytic EBV gene expression has oncolytic potential as it might destroy specifically tumor cells harboring EBV. Only little is known about how EBV contributes to tumor formation. *In vitro*, the latent genes necessary for the remarkable transforming capability of EBV are *LMP1* and *EBNA1, EBNA3B and EBNA3C*. *In vivo*, however, EBV is not equally efficient in transforming cells, reflected by the fact that 90% of the adult human population is carrier of the virus. Consequently, other factors must play important pathogenetic roles. Importantly, the malaria pathogen *Plasmodium falciparum* or HIV are known to enhance the incidence of EBV-associated malignancies.

EBV reactivation from latency or the contribution of EBV to malignancies cannot be investigated in a mouse model due to the narrow host range of EBV. Mice cannot be infected with EBV. Consequently, researchers often use MHV-68 as a surrogate. Although EBV shares great homology with MHV-68, there are differences - e.g. MHV-68 does not have transformation capability – which makes it difficult to predict the behavior of EBV in humans by extrapolating results from MHV-68 in mice. Moreover, there are distinct differences in TLR expression in mouse compared to human. In humans, only plasmacytoid dendritic cells and B-cells respond to TLR9 ligands. The limited expression of TLR9 in humans contrasts the expression in mice in which B-cells, monocytes, and probably all dendritic cells subsets express TLR9 (58, 59). Consequently, TLR9 triggering leads to a unique cytokine and chemokine expression profile, which differs between humans and mice (60). This differential TLR9 expression pattern affects the validity of the mouse model if one wants to assess experimental murine results for potential applicability in humans (51, 60). Thus, human B-cell lines are important tools for investigating the effect of the TLRs on EBV gene expression.

As it was found that TLR9 ligands strongly enhance EBV-induced immortalization of B-cells (47), and in addition, that *Plasmodium falciparum*, a factor epidemiologically linked to the development of BL, triggers TLR9 (46, 66), we aimed to investigate TLR9 in EBV-positive BL cell lines. In this work, we could show that TLR9 triggering activates several signaling pathway components including PI3K, MAPK and NF-κB, resulting in a distinct cytokine profile in BL cell lines. However, TLR9 engagement does not have a significant effect on proliferation although other reports on non-BL cell lines described enhanced proliferation or apoptosis in B-cells due to TLR9 stimulation. Nevertheless, TLR9 triggering leads to a significant suppression of EBV lytic gene expression, which could be shown at the mRNA and protein level (see manuscript 1). Moreover, TLR9 does not inhibit the initial steps of BCR signaling. That BCR signaling can be inhibited in the first signaling steps is exemplified by the interference of EBV's LMP2 with the BCR-induced Ca^{2+} flux (for detailed information see manuscript 4). However, suppression of EBV lytic reactivation is independent of the inducing agent as TLR9 suppresses both BCR- and TPA-induced EBV lytic gene expression (see manuscript 1). Importantly, although NF-κB has a well-established role in affecting gamma herpesvirus gene expression, this transcription factor seems not to be involved in the TLR9-induced suppression of lytic EBV. A milestone in this work is the finding that TLR9 affects lytic EBV gene expression in the Akata BL cell line as well as in the EBV-infected BL cell line Mutu I and in the MHV-68-infected cell line S11 (see manuscript 1). However, this general effect of TLR9 seems not to be reflected by a general mechanism. In Akata cells, histone modifications induced by TLR9 seem to be the responsible mechanism as investigated with the HDAC inhibitor Trichostatin A and the chromatin immunoprecipitation assay (see manuscript 1). As the efficiencies for gamma herpesvirus reactivation in Mutu I or S11 cells are not as high as in Akata cells, we could not determine enriched phosphorylated or acetylated histones in sufficient quantities on the lytic promoter upon BCR or TPA induction in these cell lines, respectively. Thus, two contrasting conclusions for Mutu and S11 cells can be drawn from here: First, the less efficient reactivation results in histone modifications in such a low amount that cannot be detected in a whole population of cells. Second, a different mechanism might be responsible for TLR9-induced suppression of gamma herpesvirus in Mutu I and S11 cells.

This "same-effect-but-different-mechanism" model seems not only to be applicable to cell lines infected with EBV or MHV-68. Part of the work presented here, describes the TLR9-induced suppression of EBV lytic gene expression in *ex-vivo* infected cord blood

mononuclear cells (CBMCs) (manuscript 2). This suppression was mediated in part by the TLR9-induced expression of IL12 and IFNγ. Moreover, treatment with recombinant cytokines was able to influence EBV lytic gene expression in *ex-vivo* infected cord blood mononuclear cells (CBMCs). However, we could not detect any inhibition of *BZLF1* expression in Akata cells treated with recombinant IFNγ and IL12 (data not shown). Thus, the suppression of EBV lytic gene expression upon TLR9 triggering is differentially regulated in chronically EBV-infected BL Akata cells compared to acutely EBV-infected CBMCs.

Humans encounter multiple pathogens during daily life and thus evolved multiple ways of innate and adaptive defense responses. Very successful viruses like the gamma herpesvirus Epstein-Barr virus (EBV), which establishes a life long latent B-cell infection in over 90% of the world's adult population, must have developed mechanisms in order to hide from immune responses triggered by other similar pathogens. Interestingly, a study from Martin *et al.* showed that EBV has the potential to manipulate the innate immune pathway of toll-like-receptor 7 in order to establish EBV latent infection successfully (122). We could show here that EBV lytic gene expression is substantially suppressed upon engagement of Toll-like receptor 9 (TLR9). TLR9 recognizes DNA-containing CpG, a motif found in DNA of bacteria and viruses like the herpesviruses family. Thus, EBV developed a strategy to hide from the immune responses launched by the human body against EBV-similar pathogens by staying in latency with only few genes - or none - expressed to avoid immune recognition. We also showed that the responsible mechanism involves most probably TLR9-induced histone modifications on the EBV lytic promoter *BZLF1*. Histone modifications upon TLR engagement were previously shown to occur on individual host cytokine promoters in order to prevent excessive inflammation (110). Thus, it is tempting to speculate that EBV has adapted to use a host mechanism in order to prevent its own extinction. Moreover, gamma herpesviruses in cell lines other than Akata, like Mutu (EBV) or S11 (MHV-68), seem to have developed the same strategy of avoiding immune clearance. Nevertheless, this might be achieved by different mechanisms. Thus, depending on the virus and the background of the cell, the gamma herpesviruses have developed ways to avoid clearance by TLR9-induced immune responses through apparently different mechanisms.

However, hiding in latency upon danger signals from TLRs might lead to detrimental effects in the host. By inducing suppression of EBV lytic gene expression, TLR9 might

enhance the latent state of the virus and consequently the probability of tumor formation. Chronic stimulation of TLR9 – e.g., by the malaria pathogen *Plasmodium falciparum* in endemic BL regions – might therefore be one of the key factors of BL development and reflect a situation to which EBV has not had time to adapt, yet.

The regulatory mechanisms of how TLR affects gene expression are only beginning to reveal. It is known that TLR stimulation leads to activation of transcription factors like NF-κB, AP1 or IRFs and thus regulates gene expression. Recently it was found that TLR engagement also affects the histone architecture of host cytokine promoters and thereby modulates expression of specific genes to avoid extensive inflammation. We show for the first time that TLR9 also modulates viral promoters, reflecting presumably an evolutionary mechanism of gamma herpesvirus to avoid clearance from the immune system. How the modulation of histones on some but not all promoters is achieved is currently not known. The next milestones, that need to be further investigated, are the unidentified components participating in the TLR9-induced signaling pathway leading to modifications in the histone structure. As no study identified the mechanism by which the TLR9-induced nucleosome remodeling on host promoters occurred, it can only be speculated how TLR9 induces changes in the histone architecture. TLR engagement strongly induces distinct sets of MAP kinase kinases (MKKs/MEKs) as well as MAPK phosphatases, e.g. DUSP1 (MAPK phosphatase 1) (114). These enzymes in turn are able to differentially regulate the activity of the AGC kinase family members, e.g., MSK1/2 and RSK2, which are known to affect phosphorylation of histone H3 at Ser10 (114, 115). This specific histone modification was demonstrated to occur at the *BZLF1* promoter (see manuscript 1). When it comes to acetylation of histones, TLR9 might affect the recruitment of co-activators which have histone deacetylase (HDAC) or histone acetyl-transferase (HAT) activities (like p300/CBP), leading to chromatin remodeling on host and viral promoters and subsequent changes in gene expression.

Revealing the signaling components responsible for the TLR9-induced histone modifications on the EBV *BZLF1* promoter might lead to new therapeutic approaches to counteract the TLR9-induced suppression of lytic EBV. Thus, by blocking these signaling components during chronic malaria infection, one reduces the risk of endemic BL development. This approach might also be beneficial for oncolytic-aimed therapies in already established EBV-associated malignancies. Interfering with the TLR9-induced suppression of

EBV lytic gene expression might lower the threshold for inducing EBV reactivation and thus the death of EBV-positive tumor cells.

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Curriculum Vitae

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Publication list

1) K. Ladell*, M. Dorner*, **L. Zauner**, C. Berger, F. Zucol, M. Bernasconi, F.K. Niggli, R.F. Speck, and D. Nadal. 2007. Immune activation suppresses initiation of lytic Epstein-Barr virus infection. *Cell Microbiol 9:2055-2069*; * contributed equally

2) Rechsteiner, M.P., C. Berger, **L. Zauner**, J.A. Sigrist, M. Weber, R. Longnecker, M. Bernasconi, and D. Nadal. 2008. Latent Membrane Protein 2B Regulates Susceptibility to Induction of Lytic Epstein-Barr Virus Infection. *J Virol 82:1739-1747*

3) M. Dorner, S. Brandt, M. Tinguely, F. Zucol, JP. Bourquin, **L. Zauner**, C. Berger, M. Bernasconi, RF. Speck, and D. Nadal. 2009. Plasma cell Toll-like receptor (TLR) expression differs from that of B-cells and plasma cell TLR triggering enhances immunoglobulin production. *Immunulogy Accepted Article*; doi: 10.1111/j.1365-2567.2009.03143.x

Selected Poster Presentations, Talks and Awards

1) Toll-like receptor 9 triggering inhibits Epstein-Barr virus lytic gene expression (Poster)
XIX. Meeting of the Swiss immunology PhD students, Switzerland, March 26 – 28, 2007

2) Suppression of Epstein-Barr virus (EBV) lytic gene expression in Burkitt's lymphoma (BL) following CpG ODN treatment is dependent on Toll-like receptor-(TLR) 9 yet independent of NF-κB activation and cytokine signaling (Poster)
Innate Immunity: Signaling Mechanisms, Keystone, Colorado, USA, February 24 - 29, 2008

3) Suppression of Epstein-Barr virus (EBV) lytic gene expression in Burkitt's lymphoma (BL) following CpG ODN treatment is dependent on Toll-like receptor-(TLR) 9 yet independent of NF-κB activation and cytokine signaling (Poster)
7th Day of Clinical Research, University Hospital Zurich, Switzerland, March 27, 2008

4) Toll-like receptor-9 signaling in the EBV-positive Burkitt's lymphoma cell line Akata (Talk, 1st Prize for the best student talk).
Microbiology and Immunology Student Retreat, Fiescheralp, Wallis, Switzerland, September 07 – 09, 2008

5) The Toll-like receptor 9 (TLR9)-induced suppression of Epstein-Barr virus (EBV) lytic gene expression is MyD88 dependent yet independent of NF-κB activation (Poster)
1st Swiss Workshop in Fundamental Virology, Fribourg, Switzerland, January 26 – 27, 2009

5) The Toll-like receptor 9 (TLR9)-induced suppression of Epstein-Barr virus (EBV) lytic gene expression is MyD88 dependent yet independent of NF-κB activation (Poster)
9th Charles Rodolphe Brupbacher Symposium, Targets for Cancer Prevention and Therapy, Zurich, Switzerland, February 11 – 13, 2009

6) The Toll-like receptor 9 (TLR9)-induced suppression of Epstein-Barr virus (EBV) lytic gene expression is MyD88 dependent yet independent of NF-κB activation (Poster)
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Manuscripts

1) TLR9 triggering induces suppression of EBV lytic gene expression via histone modification

Ludwig Zauner, Gregory T. Melroe, Markus P. Rechsteiner, Marcus Dorner, Martina Arnold, Christoph Berger, Michele Bernasconi, Beat W. Schaefer, Roberto F. Speck, David Nadal

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2) Immune activation suppresses initiation of lytic Epstein-Barr virus infection

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3) Plasma cell Toll-like receptor (TLR) expression differs from that of B-cells and plasma cell TLR triggering enhances immunoglobulin production

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4) Latent Membrane Protein 2B Regulates Susceptibility to Induction of Lytic Epstein-Barr Virus Infection

Markus P. Rechsteiner, Christoph Berger, Ludwig Zauner, Jürg A. Sigrist, Matthias Weber, Richard Longnecker, Michele Bernasconi, and David Nadal (2008) J. Virol., Feb;82(4):1739-47.

TLR9 triggering induces suppression of EBV lytic gene expression via histone modification [1]

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Keywords: EBV, TLR9, Burkitt's lymphoma, histone modification

Abstract

By recognizing numerous pathogen-associated DNA sequences, Toll-like receptor 9 (TLR9) causes a strong innate immune response resulting in cytokine secretion and costimulatory molecule expression. Immune activation with a TLR9 ligand was shown to suppress the initiation of EBV lytic replication in primary human B-cells. Here, we show that the suppression of EBV lytic gene expression is dependent on functional TLR9 and MyD88 signaling in the Akata cell line. Furthermore, TLR9 activation suppresses not only human but also murine gamma herpesvirus lytic gene expression, independently of the pathway involved in reactivating the virus. Finally, suppression of EBV lytic gene expression due to TLR9 activation in Akata cells involves histone modifications impacting on the activation of a viral lytic gene promoter. The suppression of EBV lytic gene expression, thus EBV hiding in latency, via histone modifications may have evolved to protect the virus against a TLR9-induced immune response.

Introduction

Epstein-Barr virus is a gamma herpesvirus infecting over 90% of the human population. Subsequent to primary infection, which largely takes place during childhood or adolescence, the virus persists in infected individuals as an asymptomatic latent infection of B-cells. Occasionally, EBV will reactivate from latency, ultimately resulting in the production of progeny viral particles, death of the host cell, and infection of new cells (1, 2). Upon initiation of lytic replication, the EBV immediate-early genes *BZLF1* (encoding the protein Zta) and *BRLF1* (encoding the protein Rta) are the first viral genes expressed. They encode transcription factors that, when expressed in sufficient quantities, lead to reactivation from latency by inducing an ordered cascade of early and late lytic EBV genes expressions (3). Notably, in cell lines infected with latent EBV, the virus can be induced to lytic infection by various stimuli like anti-human Ig, TGFβ or TPA[5] (4-7).

Toll-like receptors are key components of the innate immune system and have been implicated in the recognition of structural motifs of a number of distinct microbes that cause human diseases. The endosomal TLR9 can be triggered by synthetic ODN[6] containing unmethylated CpG, a motif found in bacterial and viral DNA (8). Signaling of TLR9 in B-cells proceeds through MyD88, which then culminates in the activation of MAPKs, NF-κB and AP-1 complexes (9). Thus, NF-κB and AP-1 complexes are enabled to translocate into the cell nucleus where they promote the expression of genes involved in B-cell activation, proliferation, and in the production of inflammatory cytokines as a part of the innate immune response against pathogens (10). Moreover, it has been shown that TLRs regulate the shape and extent of the inflammatory response through chromatin modifications at the level of individual cellular promoters (11).

EBV latent genes expression may lead to growth transformation of B-cells *in vitro* (12), and latent EBV infection is strongly associated with the development of Burkitt's lymphoma (BL), the most common cancer of childhood in equatorial Africa (13). Cell lines derived from EBV-positive BL, such as the Akata cell line or the Mutu I cell line, are instrumental to elucidate the factors that regulate the balance between EBV latency and lytic reactivation. Recently, we have shown that triggering TLR9, but not TLR1/2 or TLR7/8, results in the suppression of EBV lytic gene expression and thus reinforcement of EBV latent gene expression (14).

Here, we investigated the impact of TLR9 stimulation and its downstream signaling pathways on gamma herpesviruses. Importantly, we provide strong evidence that TLR9 suppresses EBV lytic gene expression by histone modification on a viral gene promoter. We used EBV-positive Akata and Mutu I BL cells as model systems for human gamma herpesvirus and S11 lymphoma cells for murine gamma herpesvirus.

Material and Methods

Cell culture and induction of lytic gamma herpesvirus infection

Akata (EBV-positive cell line from a Japanese BL), Mutu I (EBV-positive cell line from an African BL) and S11 (cell line from murine gamma herpesvirus-68 infected mouse lymphoma) cells were cultured in RPMI 1640 medium supplemented with L-glutamine, 10 % heat-inactivated fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin sulphate (medium and supplements from Gibco, Invitrogen Life Sciences, Basel, Switzerland) at 37 ºC in a humidified atmosphere of 5 % CO_2 in air.

For induction of gamma herpesvirus lytic gene expression, cells were seeded at 1 x 10^6 ml^{-1}. Akata cells were treated with 100 μg ml^{-1} anti-IgG (Dako A0423, DakoCytomation, Zug, Switzerland) or with 50 ng ml^{-1} of TPA (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), whereas Mutu I cells with 10 μg ml^{-1} anti-IgM (Dako A0426), or with 5 ng ml^{-1} human TGFβ (100-B-001, R&D Systems, Wiesbaden-Nordenstadt, Germany) and S11 cells with 50 ng ml^{-1} of TPA (Sigma-Aldrich).

Treatment of cells with ligands for TLR9 and inhibitors of its signaling

CpG2006 for EBV and CpG1826 for MHV-68 (both final concentration 0.5 μM) treatment preceded virus induction with anti-IgG, anti-IgM, TGFβ, or TPA for 2 h. The TLR9 inhibitor ODN IRS869 (final concentration 5.0 μM) was added very shortly, the TLR9 inhibitor chloroquine (Sigma-Aldrich) (final concentration 10 μM) was added 15 min before CpG2006 treatments. Sequences of oligodeoxynucleotide (Eurogentec, Seraing, Belgium): CpG2006 (class B): 5`-T*C*G* T*C*G* T*T*T* T*G*T* C*G*T* T*T*T* G*T*C* G*T*T*-3´ (*bases are phosphorothioate); IRS869: 5`-T*C*C* T*G*G* A*G*G* G*G*T* T*G*T*-3´ (*bases are phosphorothioate); CpG1826: 5´-T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T*-3´ (*bases are phosphorothioate).

Used signaling inhibitors were applied 1 hour before CpG2006 treatment: The InSolution™ NF-κB activation inhibitor (Calbiochem, San Diego, CA, USA) with final

concentration of 1.5 μM; the PI3K-inhibitor Ly294002 (Sigma-Aldrich) with final concentration of 20 μM; the MAPK/ERK-kinase-inhibitor PD98059 (LabForce-InvivoGen, Nunningen, Switzerland) with final concentration of 50 μM, the JNK inhibitor SP600125 (Calbiochem) with a final concentration of 20 μM, and the p38 inhibitor SB203580 (upstate, Lake Placid, USA) with a final concentration of 20 μM. Trichostatin A (Sigma-Aldrich) was applied 12h before CpG2006 treatment at a final concentration of 50 ng ml^{-1}.

RNA preparation, reverse transcription and qPCR (TaqMan)

Total RNA was isolated with RNeasy mini kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. RNA was treated with DNase (DNAfree; Ambion Europe, Huntington, Cambridgeshire, UK) for removal of residual DNA. RNA values were measured with Nanodrop-1000 (Nanodrop Technologies, Wilmington, DE, USA). RNA (1 μg) was reverse transcribed in a total volume of 20 μl with oligo dT15 (Microsynth, Balgach, Switzerland) and Omniscript Reverse Transcription kit (Quiagen). RNase inhibitor (10 units) (RNasin plus, Promega, Catalys AG, Wallisellen, Switzerland) was added to each 20 μl reaction volume. Quantitative real-time PCR (TaqMan) was performed with the ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA) in a reaction volume of 10 μl. Each reaction contained a mix of the 2x ABI-TaqMan Master Mix (Applied Biosystems), primers (Microsynth) at 300 nm each, the probe (Biosearch Technologies, Novato, CA, USA) at 200 nm, and 0.67 μl of cDNA template. The assays were cDNA specific: either the forward or reverse primer or the probe was designed to span exon-exon junctions. Specificity (DNA/cDNA) was tested using RNA before and after DNase treatment and cDNA with or without prior DNase treatment. All reactions were performed in duplicate. TaqMan data were analyzed using SDS 2.2 (Applied Biosystems). *BZLF1*, *BXLF1*, *BXLF2*, *BCRF1*, *MyD88wt*, *MyD88mutant*, *hIL10*, *IFNγ*, *TNFα*, *ORF50* mRNA expression was normalized to the housekeeping genes mRNA of *HMBS*, *GAPDH* or *18sRNA*, resulting in Δ threshold cycle (ΔC_T) values. ΔC_T values were further normalized as stated in the figures. In some cases, SYBR-Green was used instead of probes according to the manufacturer's instructions (Applied Biosystems).

Calcium-flux measurements and flow cytometry

Akata cells (1 x 10^7 ml^{-1}) were treated with CpG2006 two hours before, Fluo-3 and Fura-Red Dyes (Invitrogen) (final concentrations of 6 μM and 15 μM, respectively) were given 45 min before, and anti-IgG was given immediately before calcium-flux measurement with a FC-500 (Beckmann-Coulter, Fullerton, CA, USA). Incubation of dyes was performed in the dark at 37 °C with occasional shaking, followed by a PBS washing step. Excitation was performed at 488 nm and emission with Fluo-3 at 515-535 (FL1) and Fura Red at 665-685 (Fl3). Calcium flux was measured over 5 min as a ratio of Fluo-3 / Fura Red as previously described (15).

Immunoprecipitation and western blot analyses

Cells were harvested by centrifugation. After two washes in cold PBS, cells (5 x 10^6) from each sample were incubated on ice for 20 min in 0.5 ml of immunoprecipitation (16) lysis buffer (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 0.1 % NP-40, 10 mM b-glycerophosphate, 5 mM NaF, 1 mM PMSF, 2.5 % glycerol, and 1 complete mini protease inhibitor cocktail tablet [Roche Applied Science, Indianapolis, Ind.] per 10 ml). Cell lysates were clarified by centrifugation at 8,000 x g at 4 ºC for 10 min. Immunoprecipitation was carried out with the monoclonal BZLF1 antibody (1:20 dilution; M7005, Dako) and protein G-sepharose beads (Sigma-Aldrich, product no. P3296) at 4ºC for 24 h. After four washes in washing buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 % NP-40, and 1 mM PMSF), the immunoprecipitates were dissolved in Laemmli buffer for separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 4-12 % bis-cross-linked polyacrylamide gel (Invitrogen).

After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Protran; Schleicher & Schuell Bioscience, Keene, NH) by electroblotting at 20V for 2 h. The membranes were blocked in 5 % milk in Tris-buffered saline (TBS), probed for the appropriate antibody in TBS containing 0.1 % Tween 20, and stained with enhanced chemiluminescence western blotting detection reagents (PerkinElmer, Boston, MA) in accordance with the manufacturer's protocol.

Immunofluorescence

For indirect immunofluorescence, Akata cells (5 x 10^5) were spun down onto pre-coated Shandon Double Cytoslides (Thermo Electron Corporation, Waltham, MA) using a cytospin apparatus (Shandon Cytospin3, Frankfurt am Main, Germany). After drying for 30 min, the cells were fixed for 15 min in PBS with 3.7 % formaldehyde and permeabilized for 2 min with ice-cold methanol. The anti-BZLF1 (Zta) primary antibody (Argene Biosoft, North Massapequa, NY) was used at a 1:100 dilution. Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Invitrogen) were used at a 1:1.000 dilution. Coverslips were mounted on glass slides in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). Images were obtained with a Zeiss Axioskop 2 mot *plus* microscope using a Zeiss digital camera (AxioCam MRm) and AxioVision software version 4.4 (Carl Zeiss, Göttingen, Germany).

Chromatin immunoprecipitation (CHIP) analysis

CHIP was preformed according to the manufacture's instructions (ChIP-IT Express, Active Motif, Rixensart, Belgium). Briefly, 10 x 10^6 cells were incubated with CpG2006 2h prior to incubation with anti-IgG, anti-IgM, TGFβ or TPA for 3-5 hours. Cells were harvested and fixed by formaldehyde treatment for 10 min on a shaking platform. Fixation was stopped and cells were lysed and homogenized using an ice-cold douncer. Enzymatic shearing was performed for 12.5 min. The supernatant containing the sheared chromatin was then incubated over night on a rotating wheel with the antibody of interest and the protein G magnetic beads. Washing steps and elution of the chromatin was followed by proteinase treatment. PCR analysis at 30 to 40 cycles was done in a 2 % agarose gel. Antibodies used for CHIP: Phospho-Histone H3 (ser-10) (sc 8656-R, Santa Cruz), anti-acetyl H3 (06-599 Upstate), anti-acetyl H4 (06-598 Upstate). PCR primers for CHIP: *BZLF1* Rev (+8) 5'-CTC GAG GTG CAA TGT TTA GTG AGT TAC-3', *BZLF1* For (-222) 5'-GCT AGC GCC ATG CAT ATT TCA ACT GGG C-3', *BdRF1* and *BKRF4* (17), *Cp* (C1) (18), human *GAPDH* primers (ChIP-IT control kit, Active Motif, Belgium), *Actin* For 5'-TGC ACT GTG CGG CGA AGC-3', *Actin* Rev 5'-TCG AGC CAT AAA AGG CAA-3', *ORF50* and mouse *GAPDH* primers for S11 cells (19).

Statistical Methods

Level of significance was evaluated by an unpaired Student's *t* test if two data groups were compared. When three to five data groups were compared, Bonferroni's multiple comparison test (ANOVA) was used for selected pairs of columns.

Results

TLR9 triggering inhibits BCR-induced EBV lytic gene expression in Akata cells

In order to characterize the effect of TLR9 triggering on EBV gene expression in more detail, we stimulated Akata cells with CpG2006 prior to cross-linking of the B-cell receptor (BCR) with anti-human IgG and subsequently measured the mRNA expression of *BZLF1* at various time points by quantitative real-time PCR (qPCR). Following cross-linking of the BCR, we detected a rapid increase of *BZLF1* mRNA expression with maximal levels at 4h followed by a decrease in *BZLF1* mRNA expression over the next 20 hours (Fig. 1A), which conforms with previous reports (6, 20). After TLR9 triggering with CpG2006, a reproducible significant reduction of the induced *BZLF1* mRNA expression was observed (Fig. 1A), whereas treatment with CpG2006 alone did not result in any measurable *BZLF1* mRNA expression.

Next, we wanted to determine if the inhibitory effect of TLR9 triggering on EBV gene expression also acts on EBV late lytic genes such as *BXLF2* (gp85 protein). The mRNA expression of *BXLF2* after BCR cross-linking was strongly reduced following TLR9 triggering with a more pronounced reduction at the end of the 24h observation period (Fig. 1B). Similar observations were made with the early lytic gene *BXLF-1* (thymidine kinase TK) and the late lytic gene *BCRF1* (viral IL10) in accordance with their appearance in the lytic cascade (data not shown).

To confirm the mRNA data, we performed western blot analyses of Zta (protein of the *BZLF1* gene). To enhance the detection of Zta we performed immunoprecipitation of Zta. Triggering TLR9 reduced the BCR-induced Zta expression to undetectable levels (Fig. 1C). To see whether TLR9 triggering reduces the number of cells switching to EBV lytic gene expression or if TLR9 activation reduces Zta expression to a lower level in all BCR-induced cells, we performed immunofluorescence microscopy for the Zta protein. After cross-linking the BCR we observed 10% of Akata cells expressing detectable levels of Zta after 6 hours (Fig. 1D). Treatment with CpG2006 before BCR cross-linking led to a significant reduction in the number of cells producing observable Zta (Fig. 1D). This confirmed that Zta expression is efficiently inhibited following the triggering of TLR9.

Triggering of TLR9 in Akata cells results in an up-regulation of human cytokines, primarily IL10

Further, we tested the cytokine response by treating Akata cells with the TLR9 ligand CpG2006. After 12 hours, the mRNA levels of *hIL10*, *IFNγ* and *TNFα* were increased compared to mock treatment, whereby the anti-inflammatory cytokine *hIL10* showed the highest (20 fold) increase (Fig. 2A). Measurements of those cytokines at the protein level in the cellular supernatant confirmed that the most abundant cytokine was hIL10 (Fig. 2B). Levels of TNFα were increased to a detectable level and IFNγ was not detected. The lack of IFNγ protein might be explained by either (i) the high levels of hIL10, since IL10 is known to inhibit cytokine production at transcriptional and posttranscriptional level (21-24), or (ii) insufficient translation of the IFNγ transcripts at this time point.

TLR9-induced suppression of EBV lytic gene expression is not due to interference with BCR signaling

To characterize the effect of CpG2006 on EBV gene expression, we addressed the possibility that TLR9 triggering might interfere with the first steps in BCR signaling. BCR cross-linking leads to binding and activation of tyrosine kinases Lyn and Syk, followed by a distinct release of intracellular Ca^{2+} (25-29). Therefore, CpG ODN treatment could possibly affect cellular levels of Lyn (30), leading to an inhibition of the Ca^{2+} flux, and thus reduce *BZLF1* mRNA expression.

We investigated this possibility by examining the effects of CpG2006 on Ca^{2+} flux using flow cytometry with the fluorescent dyes Fluo-3 and Fura Red as previously described (15, 29). As expected, we detected a strong release of intracellular Ca^{2+} immediately after BCR cross-linking with anti-IgG; CpG2006 alone had no affect on Ca^{2+} release (Fig. 3 A). When TLR9 triggering preceded BCR cross-linking, the signal for Ca^{2+} release was at least as strong as following BCR cross-linking alone. Thus, TLR9 triggering does not interfere with the initial BCR cross-linking nor the first steps in the BCR signaling pathway leading Lyn activation and Ca^{2+} release.

To corroborate the conclusion above, we used 12-O-tetradecanoylphorbol 13-acetate (TPA) treatment as an alternative means of promoting EBV lytic gene expression in Akata cells. TPA is known to transiently activate the Ras-MAPK pathway through Protein Kinase C (PKC) activation (31). In Akata cells, TPA treatment leads to activation of Zta in the absence of BCR cross-linking. Indeed, triggering of TLR9 significantly inhibited TPA-induced *BZLF1* mRNA expression (Fig. 3 B). This finding supports our hypothesis that TLR9-induced inhibition of EBV lytic gene expression is not due to general interference with BCR cross-linking nor with the first steps in BCR signaling.

CpG-induced inhibition of EBV lytic gene expression requires signaling through TLR9 and MyD88

To formally prove that the CpG2006's inhibitory effects on EBV replication acts through the TLR9 pathway, we investigated the effect of two TLR9 inhibitors on *BZLF1* expression, namely IRS869 which competes with binding to TLR9 (32, 33), and chloroquine, which blocks the acidification of endosomes (34). We incubated Akata cells with IRS869 or chloroquine and CpG2006, followed by BCR cross-linking. Both TLR9 inhibitors were able to reverse the inhibitory effect of CpG2006 on *BZLF1* and *BXLF2* mRNA expression (Fig. 4A). Moreover, both TLR9 inhibitors reduced *hIL10* mRNA expression (Fig. 4A), confirming the inhibitory effects of chloroquine and IRS869 on TLR9-induced signaling.

To test if the TLR9-induced inhibition of EBV lytic gene expression requires signaling through MyD88, the only known adaptor protein for TLR9 signaling in B cells (35, 36), we stably overexpressed a dominant negative mutant form of MyD88 (a gift from J. Tschopp) in Akata cells. Overexpression was confirmed by measuring MyD88 mutant and MyD88 wild type (WT) mRNA expression (Fig. 4B). Moreover, we investigated if the MyD88 mutant form acts indeed as a dominant negative protein in Akata cells. TLR9 triggering induces translocation of NF-κB into the nucleus in a MyD88-dependent manner. As expected, we did not observe translocation of NF-κB into the nucleus in Akata cells stably expressing the MyD88 mutant in response to the TLR9 ligand (Fig. 4C). Moreover, TLR9 triggering did not increase cytokine transcripts of *hIL10* (Fig. 4D) and *TNFa* (data not shown) in the MyD88 mutant Akata cells. Thus, the MyD88 mutant Akata cells can be used to examine whether CpG2006-induced suppression of EBV lytic gene expression is MyD88 dependent. Indeed, in

Akata cells stably expressing the MyD88 mutant, the TLR9 ligand CpG did not inhibit EBV lytic gene expression of *BZLF1*, which was in clear contrast to the effects observed in Akata wild-type cells (Fig. 4D).

Taken together, TLR9-induced suppression of EBV lytic gene expression is unequivocally dependent on the TLR9-MyD88 signaling axis.

NF-κB is not fully responsible for the TLR9-mediated inhibition of EBV lytic gene expression

Triggering of TLR9 activates a number of transcription factors, including NF-κB [Fig. 4C and (10)]. Notably, NF-κB has a key regulatory role in EBV replication, i.e. activation of NF-κB inhibits gamma herpesvirus lytic replication while blocking of NF-κB leads to EBV reactivation in latently infected lymphocytes (37). Consequently, we investigated if the inhibitory effect of TLR9 triggering on EBV lytic infection is mediated by NF-κB. The *BZLF1* mRNA expression induced by BCR cross-linking was increased by co-treatment with the NF-κB inhibitor (Fig. 5A). Nevertheless, TLR9 triggering still reduced *BZLF1* mRNA expression when NF-κB activation was inhibited (Fig. 5A), indicating that NF-κB is not the main mediator of TLR9's inhibitory effect on EBV reactivation. In contrast, *hIL10* transcripts were reduced due to the NF-κB inhibitor (Fig. 5A), which speaks in favor that *hIL10* expression is indeed NF-κB dependent. Thus, although NF-κB is involved in *hIL10* and *BZLF1* mRNA expression, the inhibitory effect of TLR9 triggering on *BZLF1* mRNA expression is independent of NF-κB.

Next, we investigated if other signaling pathways activated upon TLR9 triggering might be involved in the inhibition of *BZLF1* mRNA expression, in particular the Phosphatidylinositol-3 kinases (PI3K) and the MAPK ERK pathway. Using the PI3-Kinase inhibitor Ly-294002, we could show that expression of BCR-induced *BZLF1* and *hIL10* mRNA is very strongly inhibited (Fig. 5B). Further, TLR9-induced *hIL10* mRNA expression was significantly suppressed after PI3K inhibition (Fig. 5B). PI3K is therefore important for both BCR and TLR9 signaling pathways; therefore the use of Ly294002 was not able to identify the contribution of a specific signaling molecule key in the effects observed with TLR9 on EBV.

Similar to Ly294002, treatment with the MAPK ERK inhibitor PD98059 resulted in a strong reduction of anti-IgG-induced *BZLF1* mRNA expression in Akata cells. In contrast, *hIL10* mRNA expression induced via TLR9 or BCR was not suppressed by this inhibitor (Fig. 5C). Therefore, this kinase is an important component in the anti-IgG-mediated *BZLF1* mRNA expression, yet is not involved in the CpG-induced expression of *hIL10*, and thus is very unlikely to be involved in the TLR9-mediated inhibitory effects on *BZLF1*.

Moreover, inhibition of JNK completely blocked BCR-mediated *BZLF1* expression (Fig. 5E), in contrast to inhibition of p38 (Fig. 5D). However, both components of the TLR9 signaling pathway could not be solely identified for being responsible for the TLR9-induced suppression of *BZLF1*.

In conclusion, we could demonstrate that the known signaling components of TLR9 are not solely responsible for the suppression of EBV lytic gene expression.

The histone deacetylase agent Trichostatin A rescues TLR9-induced suppression of EBV lytic gene expression

We investigated the possibility that TLR9 triggering might regulate EBV gene expression by other means than affecting transcription factor activity. We found that *BZLF1* mRNA is very stable over time (see Supplementary Figure 1), and therefore is not the mechanism of how TLR9 affects EBV gene expression.

TLRs can regulate their own inflammatory response through chromatin modifications at the level of individual host promoters (11). Importantly, the promoter region of the EBV lytic gene *BZLF1* undergoes modifications, i.e. acetylation and phosphorylation of histones upon BCR cross-linking (17, 38). These histone modifications seem to be necessary, but not exclusively responsible for inducing EBV lytic gene expression (17). Therefore, we hypothesized that TLR9's inhibitory effect on EBV activation may act by blocking acetylation and phosphorylation of histones. Using Trichostatin A, an inhibitor of histone deacetylases (HDAC) (39, 40), we investigated whether the effect of TLR9 triggering on suppression of EBV lytic gene expression can be abolished. Treating Akata cells with TSA prior to TLR9 triggering rescued expression of *BZLF1* almost completely (Fig 6A). Moreover, TSA inhibited *hIL10* mRNA expression in Akata cells (Fig. 6A). Thus, histone

modifications play a role in TLR9-induced suppression of *BZLF1* expression as well as in TLR9- and BCR-induced *hIL10* expression.

As TSA blocks the effect of TLR9 in the BL cell line Akata, we investigated if this mechanism is operative in other gamma herpesvirus-infected cell lines. Therefore, we tested the EBV-positive BL cell line Mutu I and a murine gamma herpesvirus-68 (MHV-68)-positive cell line S11. In Mutu I cells, EBV enters its lytic pathway following cross-linking of surface IgM or upon treatment with TGFβ. Notably, triggering of TLR9 significantly inhibited EBV lytic gene mRNA expression (*BZLF1*) induced by either treatment (Fig. 6B). As observed in Akata cells (Fig. 6A), TSA alone was not able to induce expression of *BZLF1* (Fig. 6B). However, TSA in combination with anti-IgM or TGFβ lead to a significant increase in *BZLF1* mRNA levels in comparison to anti-IgM or TGFβ alone (Fig. 6B). In contrast to Akata cells, triggering of TLR9 in combination with TSA and the lytic EBV-inducing agents lead to a suppression of *BZLF1* mRNA expression (Fig. 6B).

S11 lymphoma cells, infected with MHV-68, are known to enter the lytic cycle following treatment with TPA (16, 41). The MHV-68 gene *ORF50* (protein name RTA) is a homologue to the EBV immediate-early lytic gene *BRLF1* and responsible in S11 cells for initiation of the viral lytic gene expression (42, 43). When we triggered mouse TLR9 with the mouse-specific CpG1826, we detected a significant reduction in the TPA-induced *ORF50* mRNA expression (Fig. 6C), similar as for EBV. In contrast to Akata and Mutu I BL cells, TSA alone induces the lytic gene expression of *ORF50* in S11 cells (Fig. 6C) confirming published data (19). Combining TSA and TPA treatments did not show an additional increase in *ORF50* expression, probably due to full activation of lytic infection upon TSA treatment alone. However, triggering TLR9 still reduced TSA/TPA-induced lytic gene expression (Fig. 6C). Thus, in mouse S11 lymphoma cells as in human Mutu I BL cells, TLR9 is able to suppress gamma herpesvirus lytic gene expression with or without prior TSA treatment.

TLR9 signaling induces modifications in histone structure of the EBV lytic promoter *BZLF1*

To verify that TLR9 affects histone modifications on the *BZLF1* promoter and thus interferes with viral gene expression in Akata cells, we preformed chromatin

immunoprecipitation (CHIP) analyses. We confirmed that cross-linking the BCR leads to an increased amount of phosphorylated histone H3 (Ser10) and acetylated histones H4 and H3 bound to the *BZLF1* promoter in Akata cells (Fig. 7). Moreover, triggering of TLR9 prior to BCR cross-linking decreased levels of phosphorylated and acetylated histones bound to the *BZLF1* promoter (Fig. 7). This, together with the TSA experiments (Fig. 6A), strongly suggests that the TLR9-induced histone modification on the viral *BZLF1* promoter is responsible for suppression of EBV gene expression in Akata cells. In contrast, upon treatment with anti-IgM, TGFβ, or TPA alone, there was no consistent change in the amount of acetylated or phosphorylated histones bound to the *BZLF1* promoter or the *ORF50* promoter in Mutu I cells or S11 cells respectively (data not shown). Moreover, consistent and significant modifications of the chromatin structure in Mutu I cells or S11 cells on their lytic promoter sites upon TLR9 triggering could not be observed (data not shown).

Discussion

The aim of the current study was to determine the mechanism of the TLR9-mediated suppression of gamma herpesvirus lytic gene expression in Burkitt lymphoma (BL) and murine lymphoma cells. As EBV-associated lymphomas express EBV latency genes *in vivo* and EBV latency genes induce and maintain cell transformation *in vitro*, elucidating the factors that promote EBV latency will contribute to the understanding of EBV-associated lymphoma formation. Here, we demonstrate that: (i) suppression of EBV lytic gene expression following treatment with CpG2006 is mediated through TLR9 and its adaptor protein MyD88 in Akata cells, (ii) TLR9 activation suppresses not only human but also murine gamma herpesvirus lytic gene expression, independently of the pathway involved in activating lytic virus, (iii) suppression of EBV lytic gene expression due to TLR9 activation in Akata cells is independent of TLR9-elicited NF-κB activity, but involves histone modifications. Our findings argue that engagement of TLR9 and subsequent signal transduction via MyD88 may result in histone modification directly inhibiting activation of viral gene promoters. This in turn suggests that evolutionary gamma herpesviruses selection by, or adaptation to, TLR9-mediated histone modification may have contributed to the increased survival of the virus in its latent state in the host cell.

A most remarkable observation is that TLR9-mediated suppression of lytic gamma herpesvirus is maintained across species, i.e. EBV and MHV-68, in lymphoma cells harboring latent virus, suggesting a general role of innate immunity receptor signaling in controlling virus gene expression. This suppression may have fundamental consequences towards tumor formation and maintenance considering the different properties of EBV and MHV-68, as EBV has strong cell transformation capacity (12) not matched by MHV-68 (44). In recent work, we showed that TLR9-mediated suppression of EBV lytic gene expression is not restricted to latently infected lymphoma cells but can also be observed in *ex-vivo* acutely infected primary cells (14). This, together with our observation made here that the TLR9-mediated suppression takes place independently of the pathway inducing lytic virus, may indicate that TLR9-mediated suppression of gamma herpesvirus lytic gene expression is likely due to a general mechanism.

A finding of considerable relevance for the understanding of innate immune responses to pathogens is our demonstration that histone modifications on an EBV promoter in response

to TLR9 triggering is involved in the suppression of EBV lytic gene expression in Akata cells. As TLR9 and BCR triggering activates partly the same signaling components like MAPK, it is reasonable that the signaling inhibitors used here did not result in elucidating the important factors for EBV regulation. However, this implies that TLR9-mediated regulatory mechanisms are not restricted to activation of host transcription factors such as NF-κB or IRFs (interferon regulatory factors) and subsequent regulation of host genes for host defense (45), but may target viral chromatin structure and thus influence viral gene expression. Admittedly, we could show histone modifications in Akata cells, but not in Mutu I cells or murine S11 lymphoma cells. However, histone modifications in Mutu I cells or murine S11 lymphoma cells may have escaped detection due to lower efficiencies of lytic virus induction when compared to Akata cells (46). Furthermore, a recent study by Miller's lab (38) suggests that in Mutu I cells, acetylation and phosphorylation of histones on the *BZLF1* promoter upon anti-IgM/TGFβ or TPA treatment, respectively, cannot be detected because (i) too few cells are induced into the lytic cycle, and (ii) multiple copies of EBV genomes are found in the cell, but not all of them become hyperacetylated in a responsive cell. Thus, the large background of hypoacetylated histones may have blurred the assays performed here. Consequently, triggering of TLR9 might as well lead to reduction of acetylation and phosphorylation of the histones on the viral promoters in Mutu I cells and murine S11 lymphoma cells, but this escapes detection when the cells are examined as whole population. Moreover, other histone modifications than the ones investigated here could be of importance in Mutu I and S11 cells.

Based on our findings and on the findings by Foster *et al.* (11), who showed that TLR signaling induces chromatin modification in order to differentially regulate host cytokine gene expression, the effect of TLR triggering on chromatin structure might be a general mechanism by which TLRs modulate host and viral gene expression in response to a danger signal. Foster *et al.* (11) suggested that TLR-induced histone modifications on host promoters regulate cytokine expression to avoid excessive inflammation while fighting an invading pathogen. Similarly, TLR9-induced histone modifications on viral promoters, as shown here, may be beneficial for the host cells and the virus, as well. Histone modifications leading to suppression of viral lytic gene expression prevent viral replication and subsequent cell death. Nevertheless, this is beneficial for the virus as well, since survival of the host cell also ensures survival of latent gamma herpesviruses. Thus, gamma herpesviruses may have taken advantage of host innate immune mechanisms during evolution to secure their latency in danger situations for the host cell. Histone modifications leading to enforcement of the viral

latent state in the case of EBV with its unique transforming potential, however, may establish a prerequisite for the development and maintenance of EBV-associated B-cell lymphomas. This must be taken into consideration when using novel cancer treatment regimens, including TLR9 triggering (47, 48), for EBV-associated B-cell lymphomas as histone modification may interfere with antineoplastic effects, but it may also represent a therapeutic target (49).

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Footnotes

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[5] TPA, 12-O-tetradecanoylphorbol-13-acetate.

[6] ODN, oligodeoxynucleotide.

Figure Legends

Fig. 1. TLR9 triggering with CpG2006 suppresses BCR cross-linking-induced EBV lytic gene expression in Akata cells.

A–B. TLR9 triggering reduces anti-IgG induced *BZLF1* (A) and *BXLF2* (B) mRNA expression in Akata cells. Akata cells were treated with CpG2006. After 2 hours anti-IgG was added. Cells were harvested at indicated times, followed by lysis of the cells, RNA extraction, reverse transcription and qPCR. Results show mRNA transcripts of (A) *BZLF1* and (B) *BXLF2* normalized to the mRNA levels of the housekeeping gene *HMBS* and over unstimulated cells. One representative experiment in TaqMan duplicates is shown out of three independent experiments.

C. TLR9 triggering reduces anti-IgG induced Zta production. Results show Western blot for Zta protein with immunoprecipitation. Cells were treated with CpG2006 for 2h, followed by anti-IgG for 6 hours.

D. TLR9 triggering leads to less cells producing Zta. Cells were treated with CpG2006, followed by anti-IgG for 6h. Zta positive cells were detected by immunofluorescence. N = 3 independent experiments. Data are represented as mean +/- SEM. *** Level of significance was $P<0.001$ by an unpaired Student's *t* test. n.d. denotes not detectable.

Fig. 2. TLR9 triggering with CpG2006 in Akata cells leads to an increase in cytokine expression, primarily hIL10.

Cells were treated with CpG2006 for 12 hours. Results in (A) show mRNA expression of *hIL10*, *IFN*γ and *TNF*α normalized to the mRNA levels of the housekeeping gene *HMBS*. N = at least 4 independent experiments each in TaqMan duplicates. Results in (B) show protein levels of the investigated cytokines in pg ml^{-1}. N = 3 independent experiments. Data are represented as mean +/- SEM. *** Significance relative to the mock treated control was $P<0.001$ by an unpaired Student's *t* test. ** Significance relative to the mock treated control was $P<0.01$ by an unpaired Student's *t* test. n.d. denotes not detectable.

Fig. 3. TLR9 triggering does not interfere with the first steps of BCR signaling.

A. Triggering of TLR9 does not inhibit the anti-IgG induced Ca^{2+} flux. Akata cells were treated with CpG2006 for 2 hours and anti-IgG immediately before FACS measurements. Top left shows baseline measurement with mock treated cells. Top right shows cells stimulated with CpG2006. Bottom left shows stimulation with anti-IgG. Bottom right shows stimulation with CpG2006 and anti-IgG.

B. TLR9 triggering with CpG2006 reduces TPA-induced *BZLF1* mRNA expression. Akata cells were treated with CpG2006 for 2 hours before TPA treatment. Cells were harvested 24 hours after TPA treatment. Results show mRNA transcripts of *BZLF1* normalized each to the mRNA levels of the housekeeping gene *HMBS* and over mock treated cells. N = 3 independent experiments. Data are represented as mean +/- SEM. *Level of significance was P=0.0163 by an unpaired Student's *t* test.

Fig. 4. CpG2006-induced suppression of EBV lytic gene expression is TLR9 and MyD88 dependent.

A. TLR9 inhibitors IRS869 and chloroquine rescue the TLR9-induced decrease of *BZLF1* and *BXLF2* mRNA expression. Akata cells were treated with chloroquine, CpG2006 or IRS869, followed by anti-IgG. Harvesting of the cells was done 4 hours after anti-IgG treatment for *BZLF1* and *hIL10* and 24 hours after anti-IgG treatment for *BXLF2*. Results show mRNA transcripts of *BZLF1*, *BXLF2* or *hIL10* normalized each to the mRNA levels of the housekeeping gene *HMBS* and to the highest value (without TLR inhibitors). Results show at least 3 independent biological experiments each in TaqMan duplicates. Data are represented as mean +/- SEM. Bonferroni's multiple comparison test (ANOVA) was used for selected samples. Level of significance was *** P<0.001.

B-D. TLR9 ligand CpG2006 suppresses EBV lytic gene expression in a MyD88-dependent way. B) Overexpression of a MyD88 dominant negative mutant in Akata cells. Results show mRNA expression of *MyD88* wildtype (WT) and *MyD88* dominant negative mutant and in transfected and not transfected Akata cells. At least 3 independent biological experiments each in TaqMan duplicates are shown. Data are represented as mean +/- SEM. Level of significance was *** P<0.001 by an unpaired Student's *t* test. C) Akata cells transfected with

MyD88 dominant negative mutant do not show translocation of NF-κB (p65) after TLR9 triggering. Western blot of nuclear NF-κB (p65) in transfected and non transfected Akata cells after mock treatment (lane 1), after 30 min (lane 2) and 90 min (lane 3) of CpG2006 treatment. One representative experiment is shown out of three independent experiments. D) TLR9 ligand CpG2006 does not suppress EBV lytic gene expression in the MyD88 dominant negative mutant transfected Akata cells. Cells were treated with CpG2006 and anti-IgG. Transcripts of *BZLF1*, *hIL10* were normalized each to the mRNA levels of the housekeeping gene *HMBS* and over mock treated samples (mock=1). Results show at least 3 independent biological experiments each in TaqMan duplicates. Data are represented as mean +/- SEM. Bonferroni's multiple comparison test (ANOVA) was used for selected samples. Level of significance was *** $P<0.001$ and ** $P<0.01$.

Fig. 5. TLR9-induced inhibition of *BZLF1* expression is not mediated by NF-κB.

Akata cells were treated with CpG2006 and anti-IgG as described before. (A) NF-κB-activation inhibitor, (B) PI3K-inhibitor (Ly294002), (C) MAPK/ERK-kinase-inhibitor (PD98059), (D) p38 inhibitor (SB203580), or (E) JNK inhibitor (SP600125) were added 1 hour before CpG2006 treatment. Harvesting of the cells was done 4 hours after anti-IgG treatment. Results show mRNA expression levels of *BZLF1* and *hIL10* normalized each to the mRNA levels of the housekeeping gene *HMBS*. For *BZLF1* transcripts, samples with anti-IgG treatment alone were set to 1. For *hIL10* transcripts, samples with anti-IgG plus CpG treatment were set to 1. Results show at least 3 independent biological experiments each in TaqMan duplicates. Data are represented as mean +/- SEM. Bonferroni's multiple comparison test (ANOVA) performed for *BZLF1* transcripts only. Level of significance was *** $P<0.001$ or ** $P<0.01$.

Fig. 6. TLR9 triggering suppresses human and murine gamma herpesvirus lytic gene expression, whereas Trichostatin A shows a different effect the TLR9-induced viral suppression.

Cells were treated with Trichostatin A, CpG2006, anti-IgG, anti-IgM and TPA, followed by harvest of cells.

A. Trichostatin A reverses the TLR9-induced suppression of *BZLF1* expression in Akata cells. Results show mRNA expression levels of *BZLF1* and *hIL10* normalized each to the mRNA levels of the housekeeping gene *18sRNA* (as *HMBS* levels vary due to TSA treatment). For *BZLF1* transcripts, samples with anti-IgG treatment alone were set to 1. For *hIL10* samples with anti-IgG plus CpG2006 treatment were set to 1.

B. TLR9 triggering in Mutu cells suppresses EBV lytic gene *BZLF1* induced by anti-IgM or TGFβ, but Trichostatin A does not reverse the effect of TLR9. Results show mRNA expression levels of *BZLF1* normalized each to the mRNA levels of the housekeeping gene *18sRNA*. Samples with anti-IgM or TGFβ treatment alone were set to 1.

C. TLR9 triggering in S11 murine lymphoma cells with CpG1826 suppresses MHV-68 lytic gene *ORF50*, but Trichostatin A (50) does not reverse the effect of TLR9. Results show mRNA expression levels of the immediate-early *ORF50* normalized each to the mRNA levels of the housekeeping gene mouse *GAPDH*. Samples with TPA treatment alone were set to 1.

Results show at least 3 independent biological experiments each in TaqMan duplicates. Data are represented as mean +/- SEM. Statistical test used for selected samples: Bonferroni's multiple comparison test (ANOVA). *** $P<0.001$. ** $P<0.01$. * $P<0.05$.

Fig. 7. TLR9 triggering leads to decreased levels of phosphorylated and acetylated histones bound to the *BZLF1* promoter in Akata cells.

Akata cells were treated with CpG2006 and anti-IgG. Harvest of the cells and performance of CHIP assay was followed by PCR for the *BZLF1* promoter region. As controls, PCRs for various host and viral promoters regions (*Actin, GAPDH, Cp, BdRF1, BKRF4*) were performed, as well as PCR of *BZLF1* from the input DNA and a CHIP assay using a control antibody (IgG). One representative experiment is shown out of at least two independent experiments. Annotations: Lanes 1: mock treated; 2: CpG2006 treated; 3: anti-IgG treated; 4: CpG2006 and anti-IgG treated.

Supplemental Data

Supplementary Fig. 1: Transcripts of *BZLF1* are stable over time.

Akata cells were treated with CpG2006 and anti-IgG as described before. Actinomycin D (final 2µg ml^{-1}) was added after 1h after anti-IgG treatment. Transcripts of *BZLF1*, *hIL10* and *TNFα* were normalized over the housekeeping gene *HMBS* and over the starting value (t=0). Results show at least 3 independent biological experiments each in TaqMan duplicates. Data are represented as mean.

Figure 1

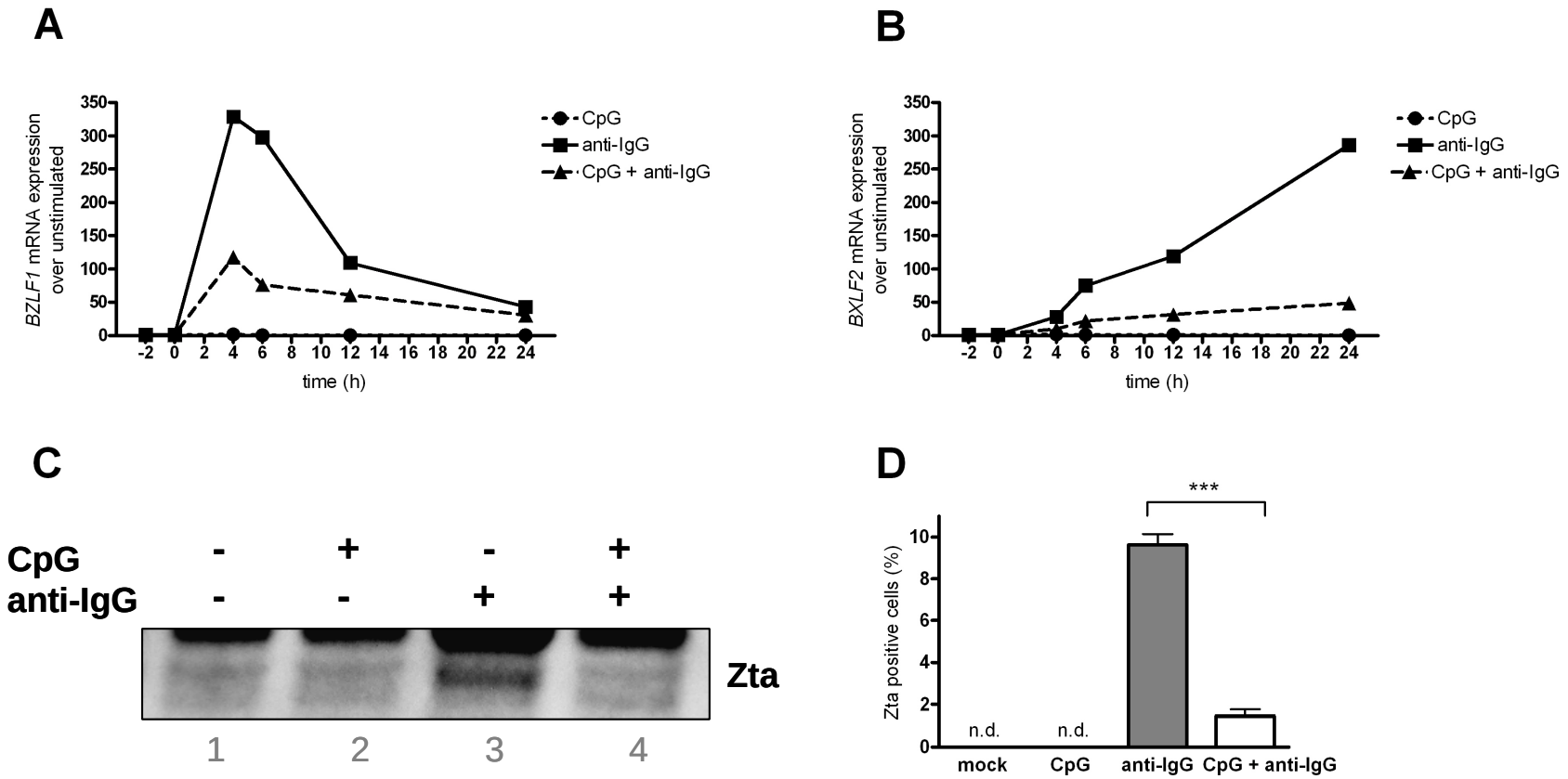

A
BZLF1 mRNA expression over unstimulated
350
300
250
200
150
100
50
0
-2 0 2 4 6 8 10 12 14 16 18 20 22 24
time (h)
CpG
anti-IgG
CpG + anti-IgG
B
BXLF2 mRNA expression over unstimulated
350
300
250
200
150
100
50
0
-2 0 2 4 6 8 10 12 14 16 18 20 22 24
time (h)
CpG
anti-IgG
CpG + anti-IgG
C
CpG - + - +
anti-IgG - - + +
Zta
1 2 3 4
D
***
Zta positive cells (%)
10
8
6
4
2
0
n.d.
n.d.
mock
CpG
anti-IgG
CpG + anti-IgG

Figure 2

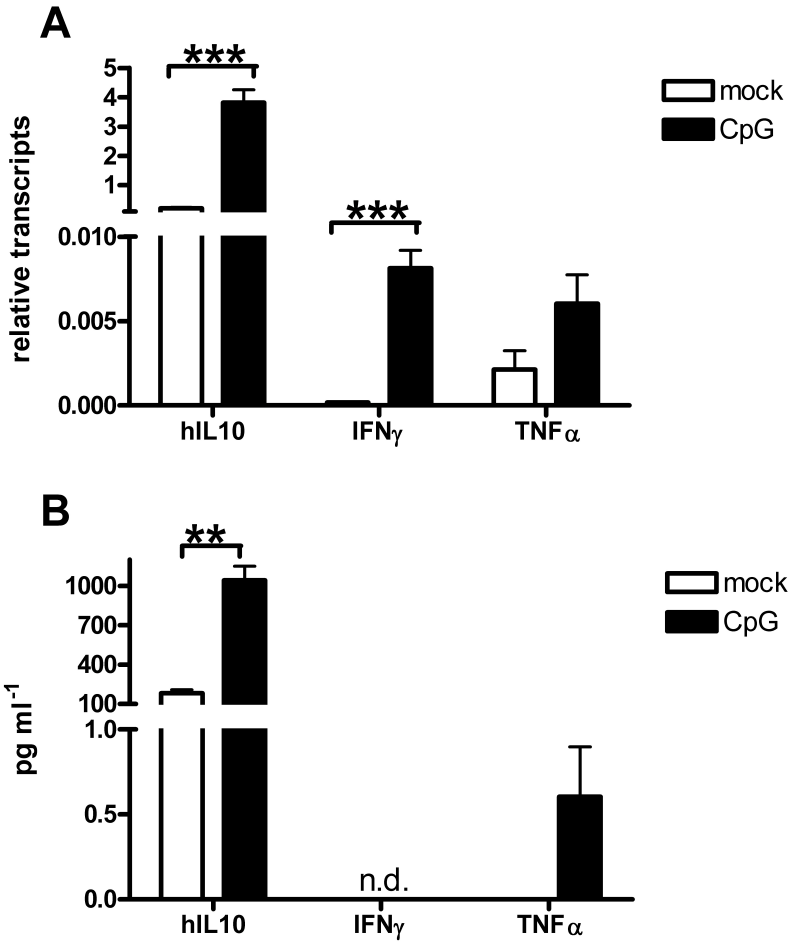
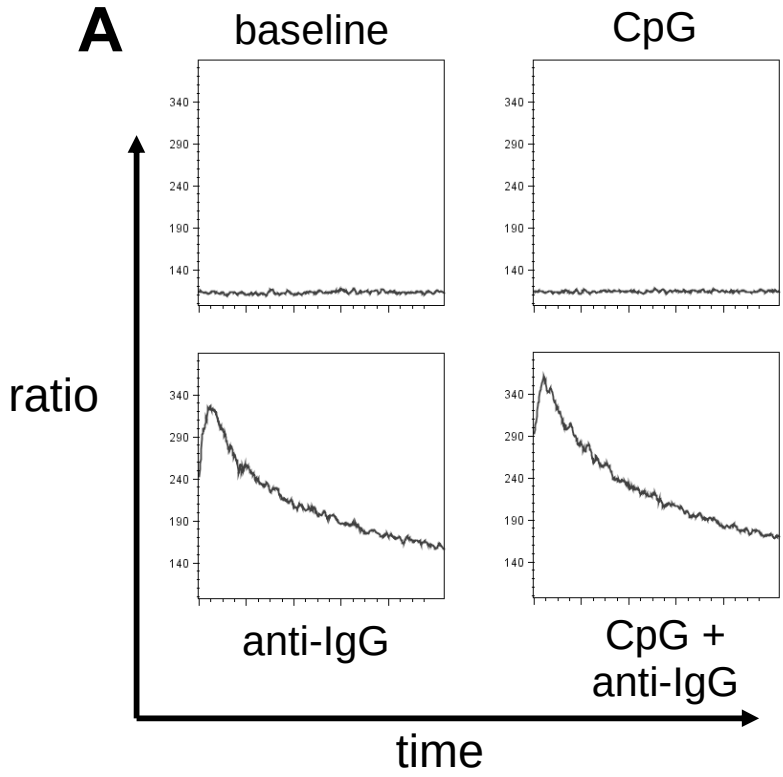

A
***
***
5
4
3
2
1
0.010
0.005
0.000
relative transcripts
mock
CpG
hIL10
IFNγ
TNFα
B
**
1000
700
400
100
1.0
0.5
0.0
pg ml-1
n.d.
mock
CpG
hIL10
IFNγ
TNFα

Figure 3


A
baseline
CpG
ratio
340
290
240
190
140
anti-IgG
CpG +
anti-IgG
time

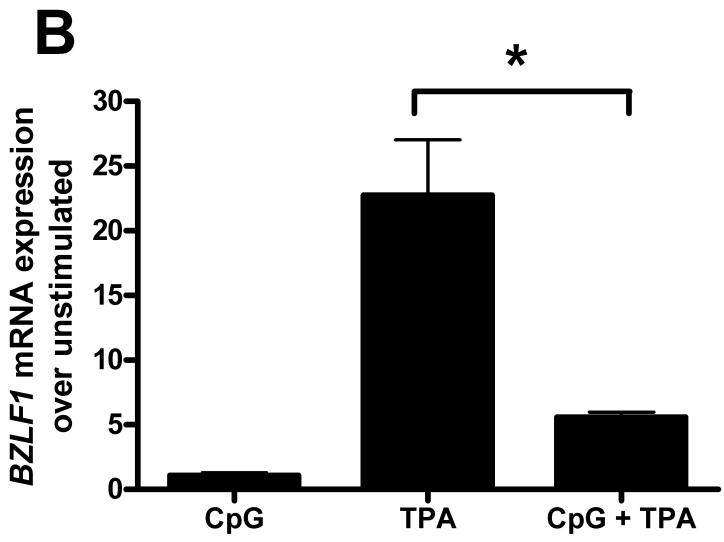

B
*
BZLF1 mRNA expression
over unstimulated
30
25
20
15
10
5
0
CpG
TPA
CpG + TPA

Figure 4

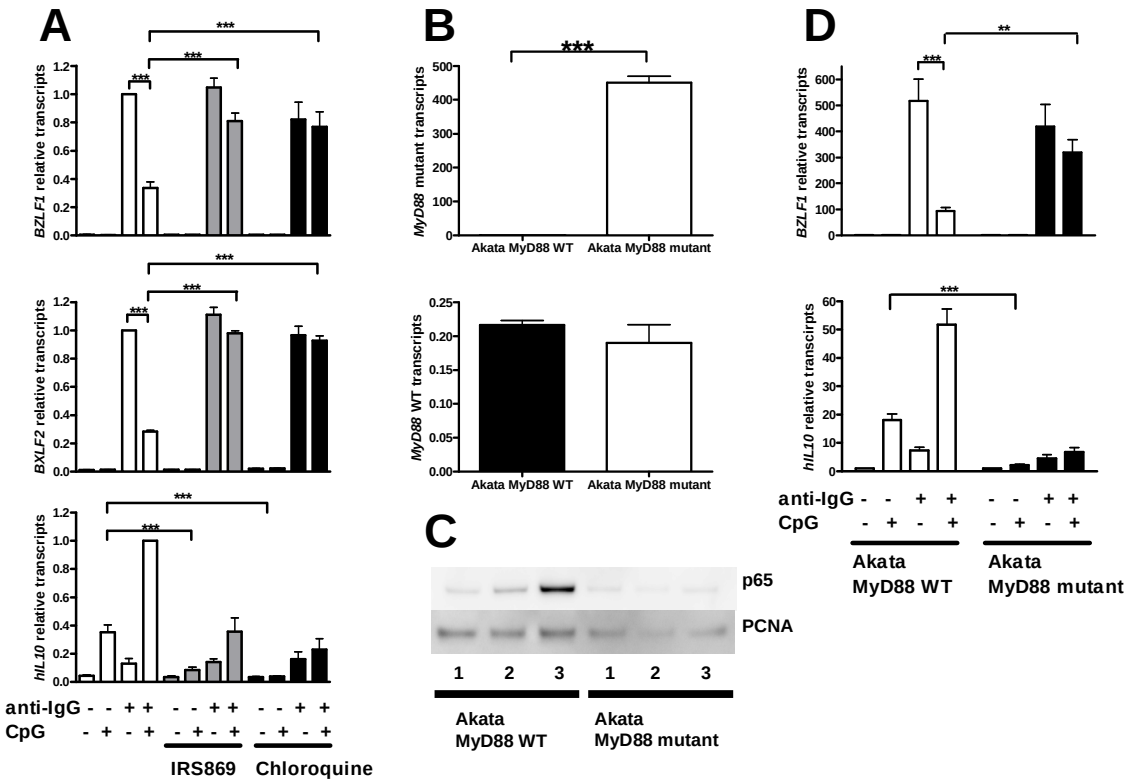

A
B
C
D
BZLF1 relative transcripts
BXLF2 relative transcripts
hIL10 relative transcripts
MyD88 mutant transcripts
MyD88 WT transcripts
Akata MyD88 WT
Akata MyD88 mutant
anti-IgG
CpG
IRS869
Chloroquine
p65
PCNA
1 2 3 1 2 3

Figure 5

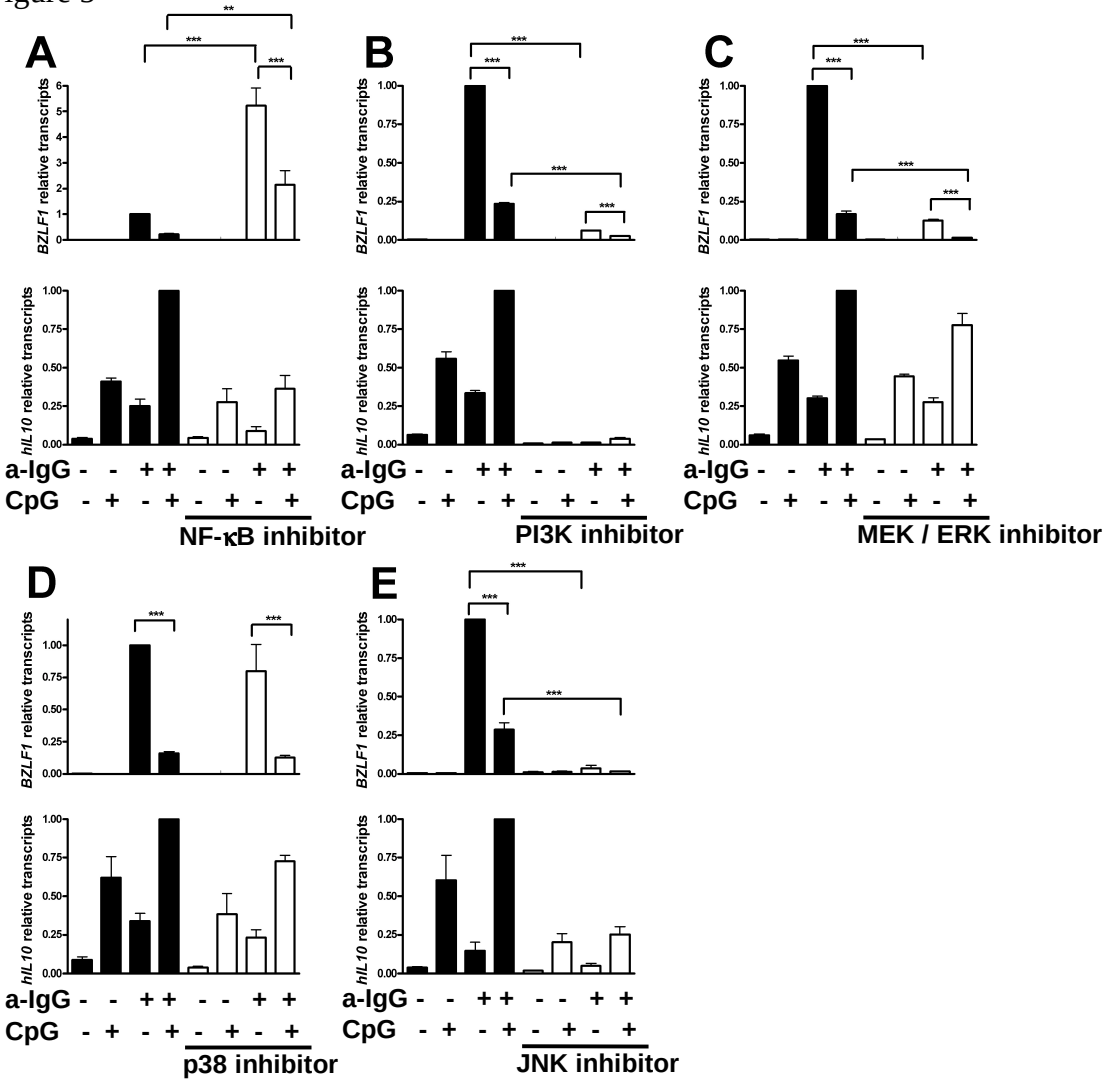

A
B
C
D
E
BZLF1 relative transcripts
hIL10 relative transcripts
a-IgG - - + + - - + +
CpG - + - + - + - +
NF-κB inhibitor
PI3K inhibitor
MEK / ERK inhibitor
p38 inhibitor
JNK inhibitor

Figure 6

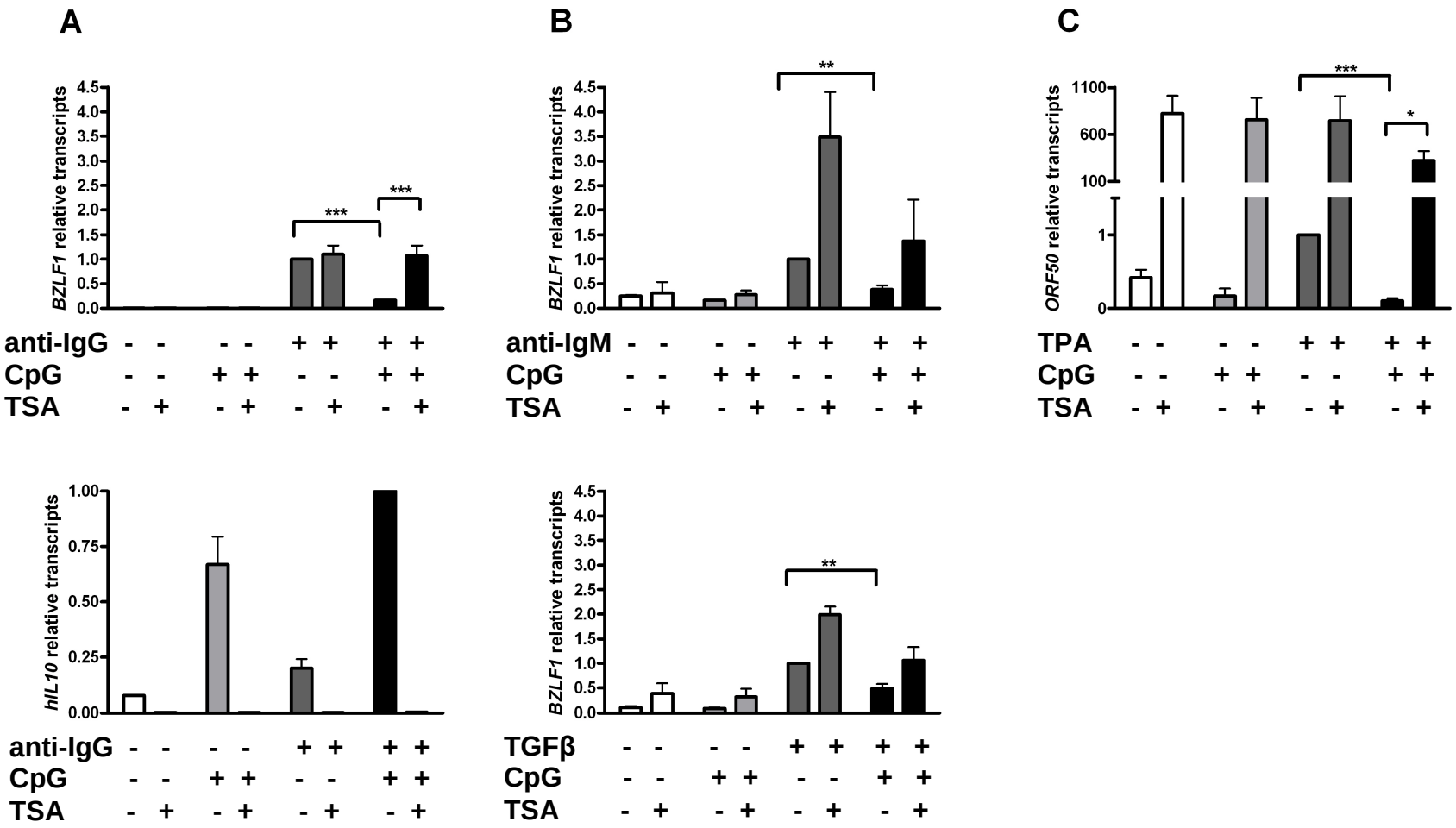
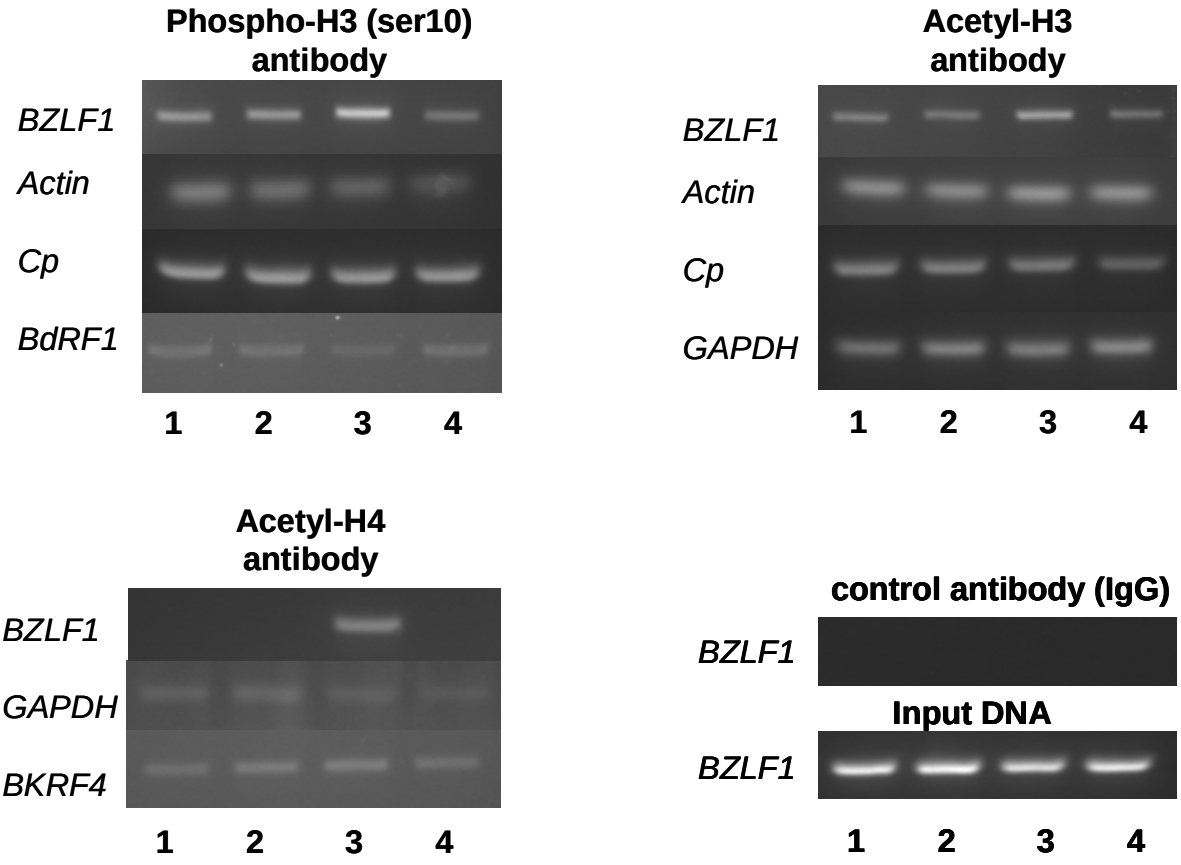

A
BZLF1 relative transcripts
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
anti-IgG - - - - + + + +
CpG - - + + - - + +
TSA - + - + - + - +
B
BZLF1 relative transcripts
**
anti-IgM - - - - + + + +
CpG - - + + - - + +
TSA - + - + - + - +
C
ORF50 relative transcripts
1100
600
100
1
0
***
*
TPA - - - - + + + +
CpG - - + + - - + +
TSA - + - + - + - +
hIL10 relative transcripts
1.00
0.75
0.50
0.25
0.00
anti-IgG - - - - + + + +
CpG - - + + - - + +
TSA - + - + - + - +
BZLF1 relative transcripts
**
TGFβ - - - - + + + +
CpG - - + + - - + +
TSA - + - + - + - +

Figure 7


Phospho-H3 (ser10) antibody
BZLF1
Actin
Cp
BdRF1
1 2 3 4
Acetyl-H3 antibody
BZLF1
Actin
Cp
GAPDH
1 2 3 4
Acetyl-H4 antibody
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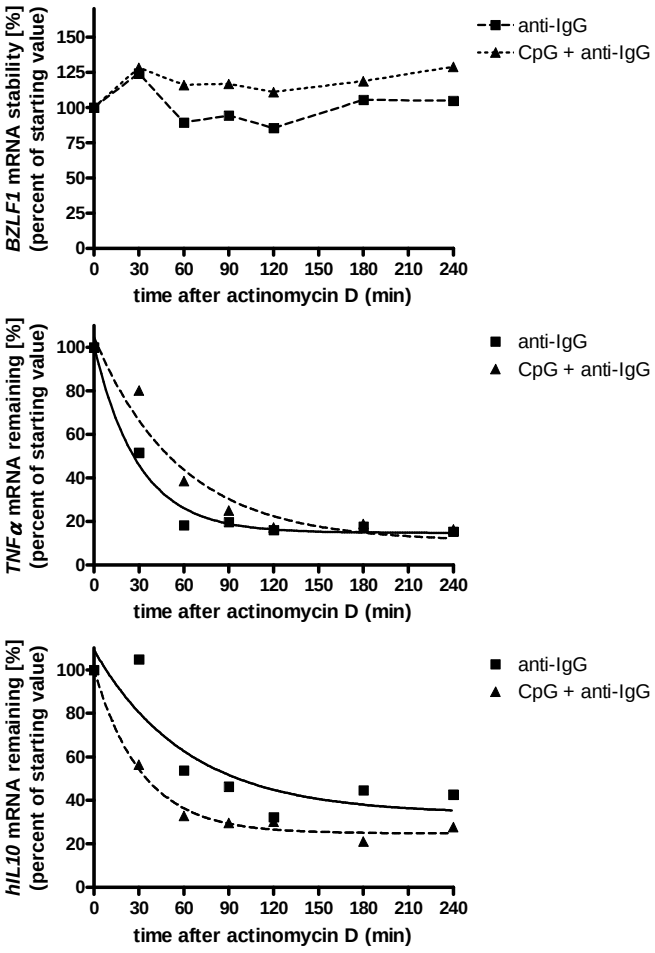
control antibody (IgG)
BZLF1
Input DNA
BZLF1
1 2 3 4

Supplementary Figure 1



TNFα mRNA half life

anti-IgG $t_{1/2}$ = 20.54 ± 4.28

CpG + anti-IgG $t_{1/2}$ = 39.71 ± 6.89

hIL10 mRNA half life:

anti-IgG $t_{1/2}$ = 43.45 ± 16.25

CpG + anti-IgG $t_{1/2}$ = 22.17 ± 4.723

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Immune activation suppresses initiation of lytic Epstein-Barr virus infection

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Summary

Primary infection with Epstein-Barr virus (EBV) is asymptomatic in children with immature immune systems but may manifest as infectious mononucleosis, a vigorous immune activation, in adolescents or adults with mature immune systems. Infectious mononucleosis and chronic immune activation are linked to increased risk for EBV-associated lymphoma. Here we show that EBV initiates progressive lytic infection by expression of *BZLF-1* and the late lytic genes *gp85* and *gp350/220* in cord blood mononuclear cells (CBMC) but not in peripheral blood mononuclear cells (PBMC) from EBV-naive adults after EBV infection *ex vivo*. Lower levels of proinflammatory cytokines in CBMC, used to model a state of minimal immune activation and immature immunity, than in PBMC were associated with lytic EBV infection. Triggering the innate immunity specifically via Toll-like receptor-9 of B cells substantially suppressed *BZLF-1* mRNA expression in acute EBV infection *ex vivo* and in anti-IgG-stimulated chronically latently EBV-infected Akata Burkitt lymphoma cells. This was mediated in part by IL-12 and IFN-γ. These results identify immune activation as critical factor for the suppression of initiation of lytic EBV infection. We hypothesize that immune activation contributes to EBV-associated lymphomagenesis by suppressing lytic EBV and in turn promotes latent EBV with transformation potential.

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Introduction

Epstein-Barr virus (EBV), a human B lymphotropic gammaherpesvirus, infects at least 90% of the world's human population. Different EBV latency gene programs allow EBV to persist in the host in latently infected B cells. Proliferation of the latently infected cells propagates EBV to the daughter cells. Latent EBV may switch to its lytic gene expression program, leading to EBV replication and subsequent lysis of the infected cell (Cohen, 2000; Rickinson and Kieff, 2001; Thorley-Lawson, 2001; Thorley-Lawson and Gross, 2004).

The vast majority of primary EBV infections occur in infants and toddlers and are usually asymptomatic (Biggar *et al*., 1978; Chan *et al*., 2001). By contrast, primary EBV infection in adolescence or adulthood may manifest as infectious mononucleosis (IM) (Biggar *et al*., 1978), with fever and enlargement of tonsils, lymph nodes, liver and spleen. This clinical presentation results from the vigorous immune activation involving proinflammatory cytokines (Foss *et al*., 1994; Chan *et al*., 2001; Rickinson and Kieff, 2001).

Epstein-Barr virus is also associated with B cell lymphoproliferative disorders, including Burkitt lymphoma, Hodgkin lymphoma, and post-transplant lymphoproliferative disease harbouring latent EBV. Infection of B cells with EBV *in vitro* in the absence of immune control is associated with B cell proliferation and transformation, indicating the oncogenic potential of EBV (Rickinson and Kieff, 2001). Immunosuppression subsequent to organ transplantation or secondary to infection with the human immunodeficiency virus increases the risk of EBV-associated lymphoproliferation (Cohen, 2000; Rickinson and Kieff, 2001; Thorley-Lawson, 2001). Also immunocompetent patients may develop Burkitt lymphomas and Hodgkin lymphomas harbouring EBV.

Burkitt lymphoma harbouring EBV is mainly seen in areas that are endemic for malaria leading to the speculation that repeated immune activation by chronic malaria or other infections is an important pathogenic factor for this tumour (Rochford *et al*., 2005). Strikingly, young

adults, experiencing IM and its vigorous immune activation to primary EBV infection, are at increased risk for EBV-positive Hodgkin lymphoma (Hjalgrim *et al*., 2003). Thus, immune activation seems to be a critical pathogenic factor in EBV-associated lymphomagenesis. The impact of activation via the innate immunity in this process is largely unknown.

Toll-like receptors (TLRs) are key players in the innate immunity. TLRs are transmembrane receptors related to the TOLL protein of *Drosophila* (Hashimoto *et al*., 1988). They are involved in the recognition of pathogens and microbial products and activate antimicrobial effector pathways (Medzhitov, 2001). Among other TLRs, B cells express TLR-9 (Hornung *et al*., 2002). TLR-9 sensors unmethylated CpG (cytosine-guanosin) dinucleotides within particular oligodeoxynucleotide sequences of microorganisms as well as the malaria pigment hemozoin (Coban *et al*., 2005). While triggering TLR-9 increases transformation rates of *ex vivo* EBV-infected B cells (Traggiai *et al*., 2004), its effect on the EBV gene expression pattern is unknown.

Based on above-mentioned epidemiological and *in vitro*

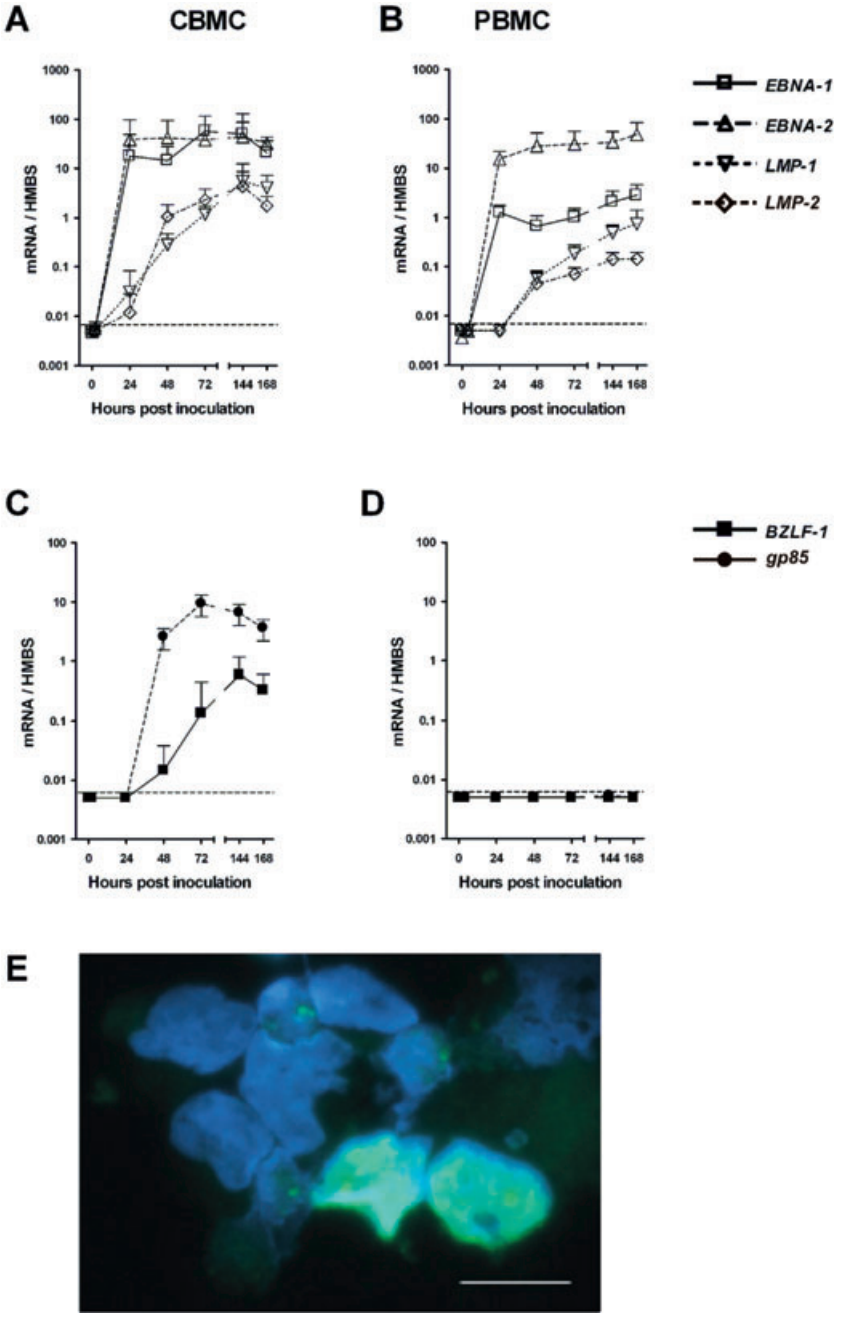

Fig. 1. Epstein-Barr virus (EBV) expresses lytic EBV mRNAs and proteins in cord blood mononuclear cells (CBMC) but not in EBV-seronegative peripheral blood mononuclear cells (PBMC) infected *ex vivo* with EBV.
A and B. mRNA expression of latent EBV genes *EBNA-1*, *EBNA-2, LMP-1* and *LMP-2* in CBMC (A) and in PBMC (B).
C and D. mRNA expression of the immediate-early lytic EBV gene *BZLF-1* and the late lytic EBV gene *gp85* in CBMC (C) and in PBMC (D).
E. Immunofluorescence of BZLF-1 protein (green) in CBMC at 240 h post inoculation with EBV.
F and G. Latent EBV infection rates in CBMC (F) and PBMC (G).
H and I. Fraction of $CD5^+$(H) and $CD5^-$ (I) B cells in CBMC showing lytic EBV at 144 h post inoculation with EBV.
mRNA expression was measured by real-time PCR and normalized to the housekeeping gene *hydroxymethylbilane synthase* (*HMBS*). Values are expressed as means ± SD induction of mRNA expression (fold) over baseline mRNA expression. No transcription was set to a value of 0.005 $\log_{10}$ as normalization to the expression of HMBS with a cycle threshold value of 40 (i.e. no transcription at cycle 40 of amplification) was always below 0.01 $\log_{10}$. The dashed lines indicate the lower limit of detection. Detection of BZLF-1 was done by indirect immunofluorescence staining in the nucleus (blue) of approximately 1 in 10^6 mononuclear cells in CBMC infected *ex vivo* with EBV. Nuclear staining with DAPI. Scale bar: 15 μm. The EBV infection rates were monitored by flowcytometry using B95.8EBfaV-GFP, a recombinant EBV encoding enhanced green fluorescent protein, and a PE-labelled anti-human CD19 antibody. Flow cytometry detection of the late lytic EBV glycoprotein gp350/220 after inoculation with B95.8 was performed using a FITC-labelled anti-EBV gp350/220 antibody, a PE-labelled anti-human CD19 antibody and a Cy5-labelled anti-human CD5 antibody to assess the susceptibility of B cell subpopulations to lytic EBV infection.

observations, we hypothesized that immune activation affects EBV gene expression. We tested our hypothesis by activating cord blood mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) from adults acutely infected *ex vivo* with EBV, and chronically EBV-infected Akata Burkitt lymphoma cells. The rationale to use CBMC was its minimal immune activation and maturity status compared with PBMC from adults (Bradley and Cairo, 2005). We avoided bias from pre-existing EBV-specific T-cell immunity by using primary cells only from EBV-naive donors.

Results

Epstein-Barr virus expresses BZLF-1 and gp85 in CBMC, but not in PBMC, after EBV infection ex vivo

We hypothesized that CBMC and adult PBMC, given their different degrees of immune activation and maturation, display distinct EBV gene expression patterns after EBV infection. Using flow cytometry, we first verified that CBMC show a lower degree of immune activation than adult PBMC by assessing the proportion of $CD4^+$ and $CD8^+$ cells expressing HLA-DR. Indeed, in CBMC ($n = 10$ donors), the percentages of $CD4^+$/HLA-DR^+ and $CD8^+$/HLA-DR^+ cells were 0.8 ± 0.5 and 0.8 ± 0.2, respectively, and in PBMC ($n = 4$ donors), they were 11.5 ± 0.4 and 20.5 ± 3.6 respectively. Next, to test our hypothesis, we quantified latent (*EBNA-1, EBNA-2, LMP-1* and *LMP-2*) and lytic (*BZLF-1,* the initiator of EBV lytic infection, and *gp85*, a late lytic gene) EBV gene mRNA expression in CBMC and adult PBMC after infection with EBV *ex vivo*. We used CBMC to model a state of immature and less vigorous immune responses than in adolescents or adults, and we used PBMC from EBV-naive individuals to prevent potential influences on EBV gene mRNA expression by pre-existing EBV-specific immunity. We measured EBV gene mRNA expression levels by real-time polymerase chain reaction (PCR) and normalized to levels of the housekeeping gene *hydroxymethylbilane synthase (HMBS)* at 0, 2, 24, 48, 72, 144 and 168 h after *in vitro* EBV inoculation of CBMC or PBMC. Similar levels of the latent genes *EBNA-1, EBNA-2, LMP-1* and *LMP-2* were detected 24 h after EBV inoculation of CBMC or PBMC (Fig. 1A and B), documenting successful infection with EBV. Levels of *EBNA-2* mRNA tended to be higher than those of *EBNA-1,* and mRNA levels of these two genes were higher than those of *LMP-1,* and *LMP-2* both in CBMC and PBMC. By contrast, significant mRNA expression of the lytic genes *BZLF-1* and *gp85* was consistently observed in CBMC (Fig. 1C) at and after 72 h post inoculation of EBV, but was never seen in adult PBMC (Fig. 1D). Accordingly, *BZLF-1* protein was detected by immunofluorescence in CBMC (Fig. 1E), but not in PBMC (not shown). It is known that EBV lytic cycle coincides with host shutoff mediated through

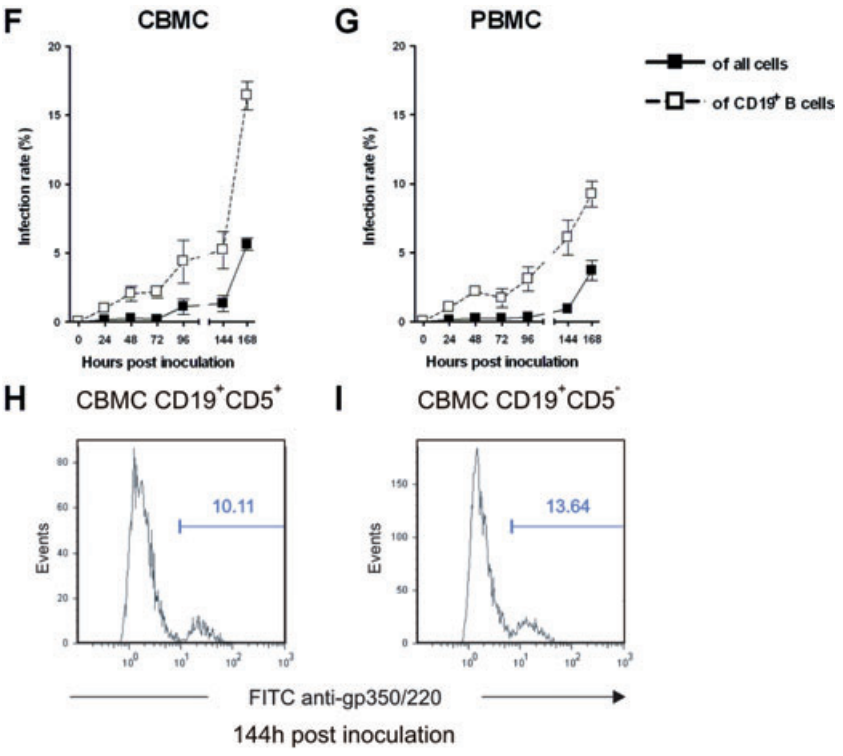

Fig. 1. *cont.*

mRNA degradation (Glaunsinger and Ganem, 2006). If that concerns the housekeeping gene *HMBS*, we used to normalize our data, this could lead to an overestimation of the lytic EBV genes. We found that the cycle threshold (Ct) values for *HMBS* mRNA expression in CBMC ($n = 6$) following *ex vivo* infection with EBV were rather constant in the first 72 h and showed a slight decrease thereafter (not shown), indicating that the abundance of *HMBS* mRNA expression is not diminishing but rather increasing.

The fractions of B cells latently infected with EBV in CBMC and PBMC following ex vivo *infection are similar, and CD5$^+$ and CD5$^-$ B cell subsets in CBMC are equally susceptible to lytic EBV*

We asked whether the difference in lytic EBV gene expression between CBMC and PBMC was due to different EBV infection rates. Thus, we estimated the fractions of latently EBV-infected B cells following *ex vivo* infection using an enhanced green fluorescent protein expressing B95.8 EBV, EBfaV-GFP (Speck and Longnecker, 1999). In separate experiments we documented that *ex vivo* infection with EBfaV-GFP resulted in qualitative and quantitative latent EBV gene expression patterns in CBMC and PBMC similar to those observed following *ex vivo* infection with B95.8 (M. Dorner *et al*., manuscript in preparation), suggesting that EBfaV-GFP is a valid substitute of B95.8. The fraction of CD19+ B cells in CBMC ($n = 3$) and PBMC ($n = 3$) at baseline was 11.6 ± 2.4% and 7.3 ± 2.3% respectively. The overall EBV infection rates were similar in CBMC and PBMC in the first 144 h after EBV inoculation *ex vivo* when they reached around 1% in relation to all cells and around 4–5% of CD19$^+$ B cells (Fig. 1F and G). To assess the fractions of lytically infected cells we stained the cells for the late lytic glycoprotein gp350/220 which is expressed on the plasma membrane (Gong and Kieff, 1990) following *ex vivo* infection with 95.8 EBV. Using flow cytometry we documented that the proportion of B cells exhibiting lytic EBV infection peaked between 2 and 3% at 144 h post EBV inoculation in CBMC while no cells expressing lytic EBV were found in PBMC (not shown). The vast majority of B cells in CBMC ($n = 3$) belonged to the CD5$^+$ B cell subset (74.17 ± 8.15%), whereas in adult PBMC the minority of B cells were CD5$^+$ (28.81 ± 11.16%). To evaluate whether the susceptibility of these B cell subsets in CBMC to lytic EBV is different, we determined the numbers of CD5$^+$ and CD5$^-$ B cells staining for gp350/220. Flow cytometry showed that the numbers of CD5$^+$ B cells in CBMC ($n = 3$) were 78 ± 5% and that the proportions of CD5$^+$ and CD5$^-$ B cells expressing gp 350/220 in CBMC ($n = 3$) were similar (11.5 ± 3.2% vs. 12.8 ± 3.8%) at 144 h following *ex vivo* infection (Fig. 1H and I), indicating comparable susceptibility to EBV lytic infection. Thus, although the fractions of B cells infected with EBV in CBMC and PBMC following *ex vivo* infection were similar, CBMC exhibited lytic EBV infection whereas PBMC did not. The expression of gp350/220 clearly indicates that the lytic infection is not only initiated (Laichalk and Thorley-Lawson, 2005) but is also fully executed in CBMC. Furthermore, the difference between CBMC and PBMC in lytic EBV gene expression cannot be attributed to the higher content of CD5$^+$ cells in CBMC than in PBMC, because CD5$^+$ and CD5$^-$ cells were equally susceptible to lytic EBV infection.

Cord blood mononuclear cells express lower levels of IL-12 p35, IFN-γ and IL-2 mRNA than PBMC at baseline and in response to EBV

Because CBMC and adult PBMC represent immune cells with dissimilar states of immune maturation with different abilities to express cytokines, we asked whether the distinct EBV gene expression in CBMC and PBMC was associated with differing cytokine gene expression. We compared mRNA levels of proinflammatory cytokines in CBMC and PBMC before and after EBV infection *in vitro*. CBMC displayed lower mRNA levels of *IL-12 p35* ($P = 0.0001$), *IFN-γ* ($P < 0.0075$) and *IL-2* ($P < 0.001$) than PBMC at baseline (Fig. 2). mRNA levels of *TNF-α*, *IL-1β*, *IL-6* and *IL-8* did not significantly differ between CBMC and PBMC at baseline (not shown).

Inoculation with EBV led to increased mRNA levels of *IL-12 p35*, *IFN-γ* and *IL-2* in CBMC and adult PBMC. However, mRNA levels in CBMC never reached the levels observed in adult PBMC, and significant differences between CBMC and adult PBMC were also found after inoculation with EBV (Fig. 3). Because host shutoff mediated through mRNA degradation during EBV lytic gene expression may result in false high positive cytokine mRNA levels, we also measured IL-12, IFN-γ and IL-2 at the protein level. At 96 h after inoculation with EBV, protein levels of IL-12 p40, IFN-γ and IL-2 were 11.4 ± 1.5 pg ml^{-1}, 6.5 ± 2.1 pg ml^{-1} and 0.8 ± 0.6 pg ml^{-1}, respectively, in CBMC supernatants ($n = 4$) versus 22.5 ± 2.3 pg ml^{-1}, 34.5 ± 7.8 pg ml^{-1} and 7.6 ± 1.7 pg ml^{-1}, respectively, in PBMC supernatants ($n = 4$). Overall, mRNA levels of *IL-12 p35*, *IFN-γ* and *IL-2* were lower in CBMC than adult PBMC before EBV infection *ex vivo* and both mRNA and protein levels of these cytokines were also lower in CBMC than adult PBMC after EBV infection *ex vivo*, suggesting that differences in expression levels of these proinflammatory cytokines may be due to maturational differences in cytokine responses and the degree of immune activation or maturation may influence the initiation of lytic EBV infection.

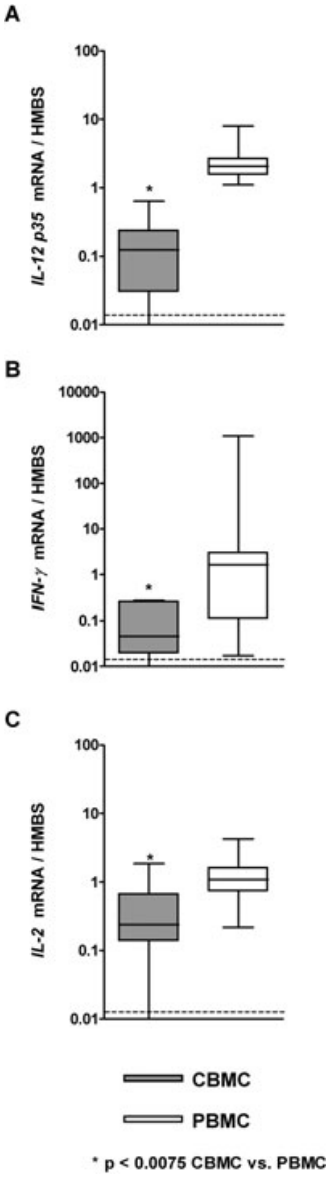

Fig. 2. CBMC express significantly lower mRNA levels of proinflammatory cytokine genes than PBMC at baseline.
A. IL-12 p35.
B. IFN-γ.
C. IL-2.
CBMC ($n = 19$) and PBMC ($n = 20$) were isolated by density-gradient centrifugation. RNA was extracted from cell pellets and treated with DNase to remove residual genomic DNA. The mRNA was reverse transcribed into cDNA using an oligo-d$(T)_{15}$ primer. mRNA expression was measured by real-time PCR and normalized to the housekeeping gene HMBS. Median values (solid black line) of fold mRNA expression in relation to HMBS are shown as box plots with whiskers that extend to the highest and lowest values above and below the box. The dashed lines indicate the lower limit of detection. * refers to CBMC versus PBMC.

The lower levels of IL-12, IFN-γ and IL-2 in CBMC than in PBMC in response to EBV are not associated with higher levels of TGF-β *or* IL-10 *mRNA*

To explore whether lower levels of proinflammatory cytokines in CBMC than PBMC after infection with EBV were associated with higher mRNA levels of anti-inflammatory cytokines in CBMC than in PBMC, we measured mRNA expression of the anti-inflammatory cytokine genes *TGF-β* and *IL-10*. Importantly, the assay we used to detect human *IL-10* is highly host specific and does not detect EBV-encoded viral *IL-10*. At baseline, mRNA levels of *TGF-β* were lower whereas levels of *IL-10* were higher in CBMC than in PBMC (Fig. 4). Following infection with EBV, levels of *TGF-β* remained lower in CBMC compared with in PBMC. By contrast, levels of *IL-10* became significantly lower in CBMC than in PBMC after EBV inoculation *in vitro* (Fig. 4). These findings strongly indicate that the lower levels of induction of IL-12, IFN-γ and IL-2 in CBMC than in PBMC after inoculation with EBV are not due to higher expression of *TGF-β* or *IL-10* mRNA in CBMC than in PBMC.

To investigate the effects of IL-10 on lytic EBV infection, we treated CBMC ($n = 3$) with recombinant human IL-10 at 1, 10, or 100 pg ml^{-1}. Adding IL-10 did not result in significant changes in *BZLF-1* mRNA expression following EBV infection *ex vivo* (not shown). Similarly, neutralizing IL-10 in PBMC from EBV-seronegative adults ($n = 3$) with anti-human IL-10 antibody at 1.0 U ml^{-1} did not result in *BZLF-1* mRNA expression following EBV infection *ex vivo* (not shown). Thus, *IL-10* levels appear to have no effect on EBV lytic gene expression patterns.

rIL-12 and rIFN-γ decrease BZLF-1 *mRNA expression in CBMC during EBV infection* in vitro

Next, we asked whether IL-12 or IFN-γ have an effect on *BZLF-1* expression in CBMC after infection with EBV. We infected CBMC with EBV *ex vivo*, treated with rIL-12, rIFN-γ or both, and measured mRNA expression 96 h after infection when *BZLF-1* is detectable in all of the EBV-infected untreated CBMC cultures (Fig. 5). In CBMC treated with rIL-12 simultaneously with EBV inoculation and then every 24 h, *IL-12 p35* mRNA expression was unchanged, whereas *IFN-γ* mRNA expression increased about 6.5-fold at 96 h, and *BZLF-1* mRNA expression level was reduced by 50%, compared with untreated CBMC (Fig. 5). As expected, treatment of CBMC with rIL-12 also increased the IFN-γ protein concentration in the cell-free supernatant of CBMC compared with no treatment (1368 vs. 37 pg ml^{-1}). This higher increase in protein concentration compared with the increase in mRNA expression may be explained by either accumulation of transcribed protein in the super-

natant or expressed mRNA being transcribed to protein at higher rates, or both. Treatment of CBMC with rIFN-γ, simultaneously with EBV inoculation and then every 24 h, did not change *IFN-γ* mRNA expression and did not influence *IL-12 p35* mRNA expression, but reduced *BZLF-1* mRNA expression by 50% compared with

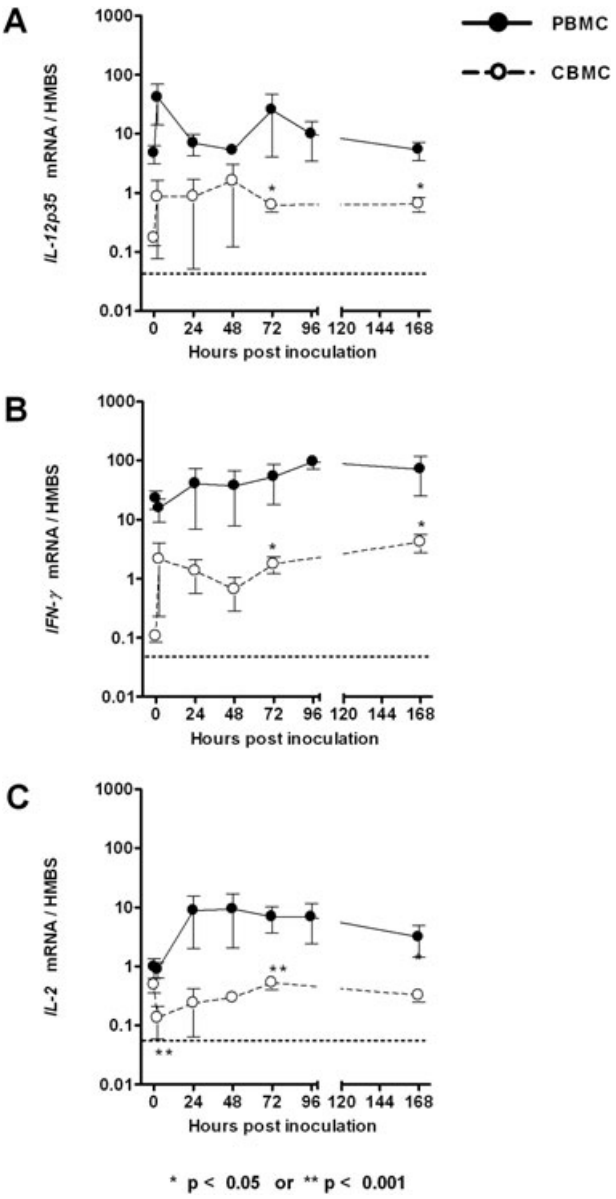

Fig. 3. CBMC express significantly lower mRNA levels of proinflammatory cytokine genes than PBMC from EBV-seronegative adults following infection with EBV *ex vivo*.
A. IL-12 p35.
B. IFN-γ.
C. *IL-2*.
mRNA expression was measured by real-time PCR and normalized to the housekeeping gene HMBS. Results are means ± SD of mRNA expression normalized to HMBS (fold) during 7 days of culture. The dashed lines indicate the lower limit of detection. * or ** refers to CBMC ($n = 3$–8) versus PBMC ($n = 3$).

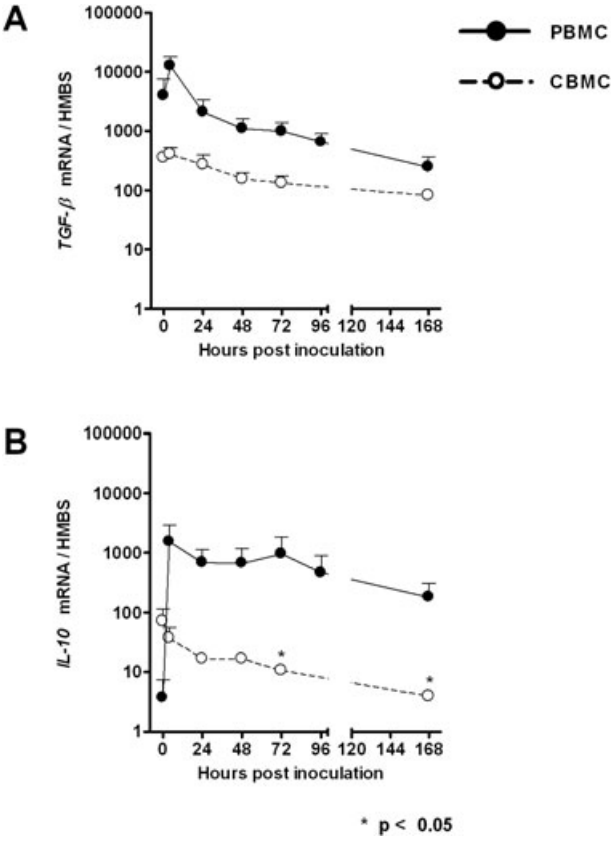

Fig. 4. CBMC express lower mRNA levels of anti-inflammatory cytokine genes than PBMC from EBV-seronegative adults in response *ex vivo* infection with EBV.
A. TGF-β.
B. IL-10.
mRNA expression was measured by real-time PCR and normalized to the housekeeping gene HMBS. Results are means ± SD of mRNA expression normalized to HMBS (fold) during 7 days of culture. * refers to CBMC ($n = 3$–8) versus PBMC ($n = 3$).

untreated CBMC (Fig. 5). Finally, treatment with both rIL-12 and rIFN-γ simultaneously with EBV inoculation and then every 24 h, did not change *IL-12 p35* mRNA expression, increased *IFN-γ* mRNA expression around 17-fold, and resulted in a stronger suppression (sixfold) of *BZLF-1* mRNA expression than when treatment included only one of both cytokines (Fig. 5). Conversely, we treated adult PBMC infected with EBV *ex vivo* with antibodies to IL-12 and IFN-γ and could not provoke *BZLF-1* mRNA expression (not shown). Thus, substitution of the proinflammatory cytokines IL-12 and IFN-γ partially suppressed *BZLF-1* mRNA expression in CBMC infected with EBV *ex vivo*, indicating that the weaker proinflammatory immune response in CBMC contributes to the initiation of lytic EBV infection seen in CBMC. The failure to provoke *BZLF1* mRNA expression in acutely infected PBMC with antibodies to IL-12 and IFN-γ together with their incomplete suppression of *BZLF-1* mRNA expression suggests that these two cytokines are

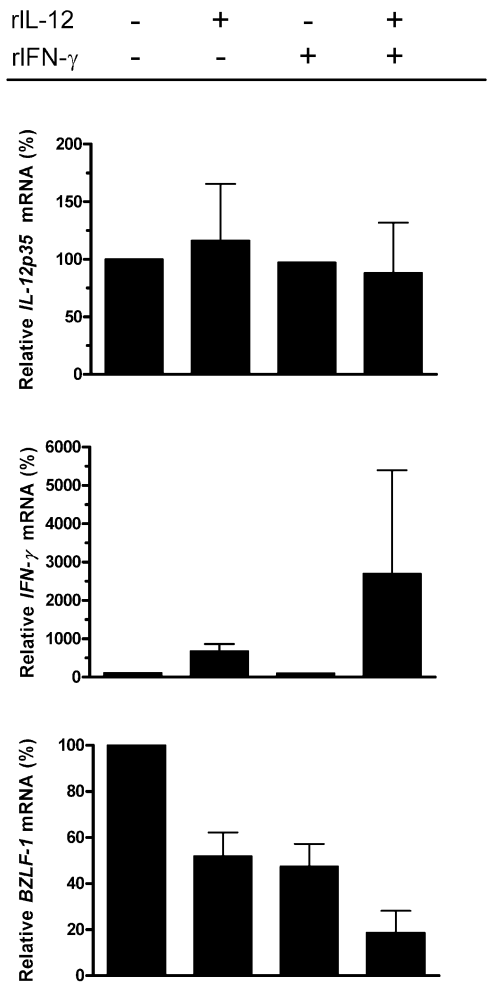

Fig. 5. rIL-12, rIFN-γ, or both suppress the transcription of *BZLF-1* in CBMC infected with EBV *ex vivo*. rIL-12, rIFN-γ, or both were added together with EBV and then every 24 h over 96 h to the cultures. mRNA was measured by real-time PCR. Results shown are from one representative experiment of six from CBMC from different donors. RNA was extracted and analysed from four different cell pellets per condition, except for the treatments with rIFN-γ (two cell pellets per condition). Means ± SD represent the differences of mRNA expression between treated and untreated samples after normalization to the housekeeping gene HMBS.

not the only players suppressing the initiation of lytic EBV infection.

CpG ODN 2006 suppress BZLF-1 *mRNA expression in CBMC infected with EBV* in vitro

We next sought to test whether other means of immune stimulation would lead to suppression of *BZLF-1* expression in CBMC. To stimulate CBMC we used the unmethylated CpG-containing ODN 2006 that triggers the innate pathogen-associated molecular pattern recognition receptor TLR-9 which is also expressed in B cells (Hornung *et al*., 2002) and exerts a proinflammatory effect (Peng, 2005). We asked if stimulating CBMC with CpG ODN 2006 alters EBV gene expression. Thus, we cultured CBMC with or without CpG ODN 2006 and with or without EBV for 96 h respectively. Treatment of uninfected CBMC with CpG ODN 2006 resulted in a 2.6-fold increase of *TLR-9* mRNA expression versus no treatment (Fig. 6A). EBV infection by itself led to 3.7-fold increase in levels of *TLR-9* mRNA expression in CBMC over uninfected CBMC (Fig. 6B). The large number of CpG motifs in the EBV DNA genome or a CpG-motif-independent mechanism may explain the upregulation of *TLR-9* by EBV. Treatment of EBV-inoculated CBMC with CpG ODN 2006 resulted in a further but not significant increase of *TLR-9* mRNA expression (Fig. 6B). As expected, inoculation of CBMC with EBV resulted in marked expression of *BZLF-1* mRNA. By contrast, EBV-infected CBMC cultures treated with CpG ODN 2006 exhibited a 5.8-fold lower *BZLF-1* mRNA expression (Fig. 6C). Although EBV itself induced *TLR-9* mRNA expression in CBMC, the induction did not suppress *BZLF-1* mRNA expression. Therefore, the reduction of *BZLF-1* mRNA expression in EBV-infected CBMC after CpG ODN 2006 treatment did not seem to depend on induction of TLR-9, but rather on the additional stimulation by CpG ODN 2006 (e.g. increased TLR-9 signalling mediated by CpG binding). Expression levels of latent EBV gene mRNAs were not significantly different in untreated or CpG ODN 2006-treated EBV-infected CBMC (not shown).

Next, we asked whether triggering of other TLRs present on B cells also results in suppression of lytic EBV. Triggering of TLR-1/2, TLR-4, or TLR-7/8 on EBV-infected CBMC did not result in significant suppression of *BZLF-1* and *gp85* mRNA expression and gp350/220 expression compared with controls (Fig. 6D–F). These data suggest that CpG ODN 2006 stimulation of TLR-9 on EBV-infected CBMC is rather specific in inhibiting the mRNA and protein expression of EBV genes involved in lytic infection but has no effect on latent EBV gene mRNA expression. This suppression of the initiation and completion of lytic EBV infection in turn may support maintenance of EBV latency.

Antibodies to IL-12 and IFN-γ partially restore BZLF-1 *mRNA expression in EBV-infected CBMC treated with CpG ODN 2006*

We next explored whether IL-12 and IFN-γ contribute to the suppression of *BZLF-1* mRNA expression induced after TLR-9 triggering by CpG ODN 2006. We added antibodies to IL-12 and IFN-γ to cultures of CBMC inoculated with EBV and treated with CpG ODN 2006. Indeed, treatment with anti-IL-12 and anti-IFN-γ partially restored expression of *BZLF-1* mRNA in these CBMC

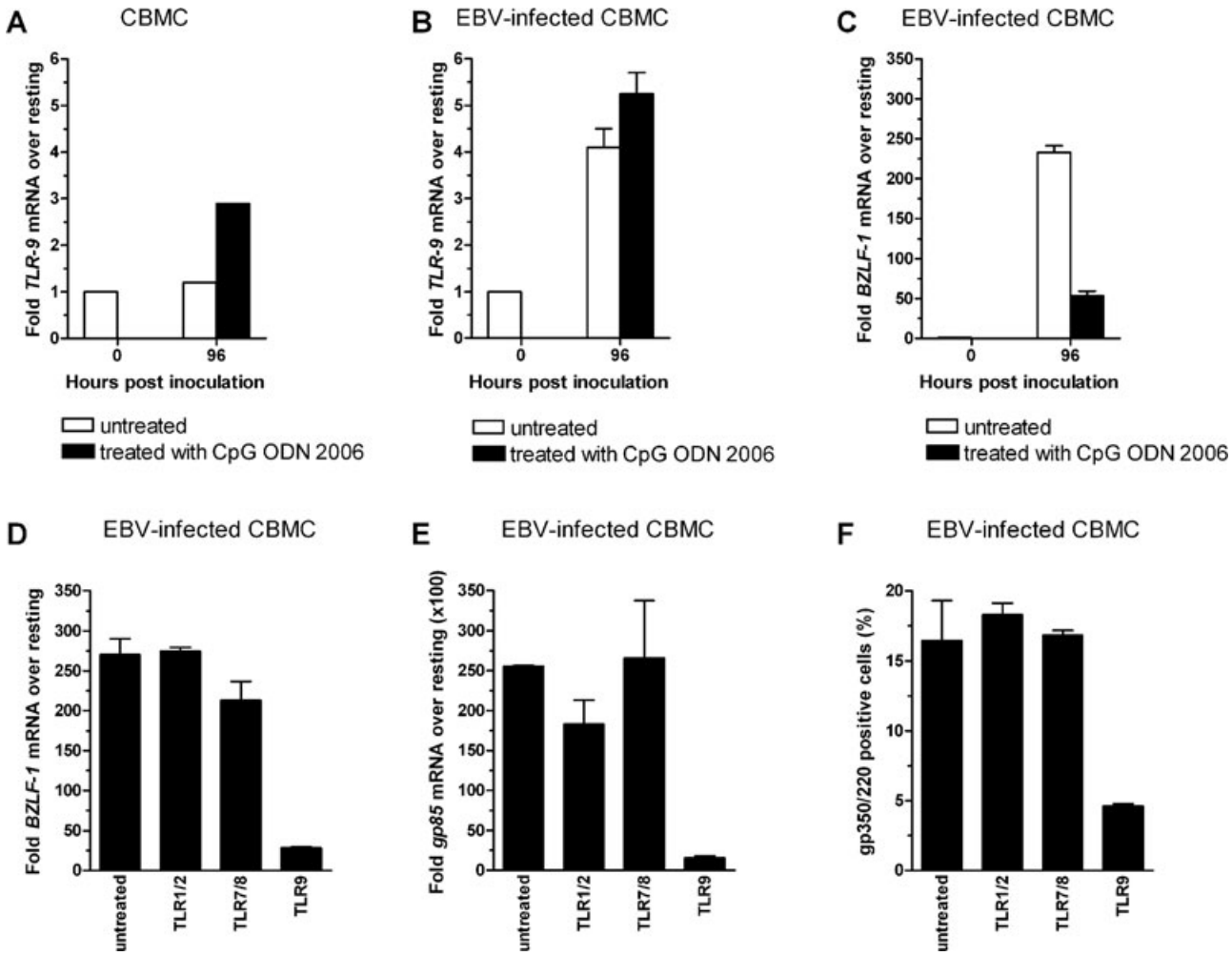

Fig. 6. CpG ODN 2006 specifically suppresses initiation and execution of lytic EBV in CBMC following *ex vivo* infection.
A. mRNA expression of *TLR-9* in CBMC ($n = 3$) treated or not with CpG ODN 2006.
B. mRNA expression of *TLR-9* in CBMC ($n = 3$) infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
C. mRNA expression of *BZLF-1* in CBMC ($n = 3$) infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
D and E. mRNA expression of *BZLF-1* (D) and EBV *glycoprotein* (*gp) 85* (E) in CBMC ($n = 3$) that were stimulated with ligands of TLRs present in B cells and infected *ex vivo*.
F. Expression of the lytic EBV glycoprotein gp350/220 in CBMC treated with ligands of TLRs present in B cells and infected *ex vivo*.
TLR ligands were added at 0 h and 90 h to 2×10^6 CBMC ($n = 3$) infected *ex vivo* with EBV. Cells were collected at 96 h. Concentrations of TLR ligands were 10 μg ml^{-1} for peptidoglycan (TLR-1/2), 3 μM for R-848 (TLR-7/8) and 1 μM for CpG ODN 2006 (TLR-9). RNA was extracted from two cell pellets per condition, treated with DNase, and reverse-transcribed into cDNA with an oligo-dT15 primer. mRNA expression was measured in duplicate by real-time PCR. Means ± SD of fold induction over resting normalized to the housekeeping gene HMBS.
Flowcytometry was performed using a FITC-anti-EBV gp350/220 antibody. Events shown are gated for CD19$^+$ B cells. One representative experiment of three is shown.

(Fig. 7A). Even though these antibodies exhibited little effect on CpG ODN 2006-induced enhanced *IL-12 p35* and *IFN-γ* mRNA expression (Fig. 7B and D), the protein levels of both cytokines were below the lower limit of detection (Fig. 7C and E). These data at the protein level excluded potentially misleading results due to host shutoff mediated through mRNA degradation during EBV lytic gene expression. Thus, our observations provide evidence that part of the negative impact on the initiation of lytic EBV infection in CBMC exhibited by CpG ODN 2006 through TLR-9 triggering is mediated by IL-12 and IFN-γ.

A key question is which cells are implicated in the effects observed. Thus, we infected highly purified B cells from CBMC and PBMC with EBV. Indeed, *BZLF-1* was expressed in B cells from CBMC but not from PBMC. mRNA and protein levels for IL-12 and IFN-γ were strikingly lower in B cells from CBMC than from PBMC (Fig. 7F–O). Moreover, we could reproduce the inhibitory effects on *BZFL-1* expression when triggering TLR-9

similar as outlined above. TLR-9 triggering was associated with an increase in IL-12 and IFN-γ at the mRNA as well as protein level. Thus, the effects we observed when triggering TLR-9 are rather direct than indirect.

CpG ODN 2006 suppresses induction of BZLF-1 *mRNA expression in Akata Burkitt lymphoma cells*

The above experiments addressed the effect of immune stimulation triggered by cytokines and TLR-9 on the initiation of lytic EBV infection in cells exposed to acute infection with EBV, but not in cells with chronic latent EBV infection. Switching from latent to lytic EBV infection may occur spontaneously or be provoked in EBV-transformed cells by several agents *in vitro* (Kieff and Rickinson, 2001). Cells from the Burkitt lymphoma cell line Akata can readily be provoked to switch from latent to lytic EBV infection within hours by cross-linking their surface IgG using anti-IgG antibodies. Thus, we asked whether triggering of TLR-9 exhibits an effect on the induction of lytic EBV infection in Akata cells, used as a surrogate for Burkitt lymphoma cells. We first determined whether Akata cells express TLR-9. Using quantitative PCR, we demonstrated that Akata cells constitutively express *TLR-9* mRNA. Stimulation of Akata cells with CpG ODN 2006 did not increase *TLR-9* mRNA expression (Fig. 8A). This suggested that TLR-9 expression in the fully differentiated Akata cells was maximal before treatment with CpG ODN 2006 as opposed to CBMC which contain naive B cells and showed an increase in *TLR-9* mRNA expression upon stimulation with CpG ODN 2006 (Fig. 6A). As expected, cross-linking of surface IgG after treatment with anti-IgG provoked the expression of *BZLF-1* mRNA and thus the initiation of lytic EBV infection (Fig. 8B). Treatment of Akata cells with CpG ODN 2006 before treatment with anti-IgG reduced *BZLF-1* mRNA expression provoked by surface IgG cross-linking by 50% (Fig. 8B). By contrast, treatment with CpG ODN 2006 simultaneously or deferred to anti-IgG treatment had no significant effect on the initiation of lytic EBV infection (not shown); indicating that the signalling cascade initiated by anti-IgG appears to be dominant to the intracellular changes subsequent to triggering TLR-9. These data suggest that triggering innate immunity via TLR-9 suppresses the initiation of lytic EBV infection in transformed B cells with established EBV latency and that this suppression is independent from other immune cells expressing TLR-9.

Discussion

Immune activation may be a critical factor in EBV-associated lymphomagenesis. In this work, we examined the effect of immune activation on EBV gene expression. We found that (i) EBV expresses *BZLF-1*, the initiator of lytic EBV infection, and the late lytic genes *gp85* and gp350/220 in CBMC, but not in adult PBMC infected *ex vivo* with EBV, (ii) lower levels of proinflammatory cytokines in CBMC than in adult PBMC are associated with expression of lytic EBV genes and (iii) triggering of TLR-9 suppresses lytic gene expression in CBMC acutely infected *ex vivo* with EBV and in anti-IgG-stimulated chronically infected Akata Burkitt lymphoma cells. Our findings, indeed, identify immune activation as critical factor for the suppression of lytic EBV infection.

We used CBMC to model a state of minimal immune activation compared with PBMC from adults. Importantly, by using primary cells only from EBV-naive individuals, we avoided bias from pre-existing EBV-specific T-cell responses, which may be triggered by *ex vivo* EBV infection. In CBMC, *BZLF-1* and *gp85* mRNA expression and gp350/220 protein expression showed a sharp rise after *ex vivo* EBV infection that persisted over the entire observation time. In adult PBMC, no *BZLF-1*, *gp85* or gp350/220 expression was seen at all, although the fractions of B cells infected with EBV following *ex vivo* infection were similar in CBMC and PBMC. The difference in lytic EBV gene expression cannot be attributed to the higher content of $CD5^+$ cells in CBMC than in PBMC, because $CD5^+$ and $CD5^-$ cells exhibited lytic EBV equally. By contrast, mRNA expression patterns of latent EBV genes were similar in CBMC and PBMC. Extending data published by Hunt *et al*. (1994) the proinflammatory cytokines IL-12, IFN-γ and IL-2 were lower in CBMC than in PBMC before EBV infection. Furthermore, levels of these cytokines in CBMC did not increase to the levels seen in PBMC in response to EBV. Based on these data, we hypothesized that the higher levels of proinflammatory cytokines in PBMC may result in the suppression of *BZLF-1* expression (i.e. that differences in the status of immune activation/maturation are responsible for the profound difference in EBV gene expression between CBMC and PBMC).

The main sources of IL-12 are monocytes and dendritic cells (DCs) (Trinchieri, 2003). As mentioned above, CBMC produce less IL-12 than PBMC (Hunt *et al*., 1994), and DC derived from neonatal monocytes transcribe much less IL-12 p35 than adult monocytes (Goriely *et al*., 2001). IFN-γ produced by natural killer (NK) cells (Biron *et al*., 1999) may, in part, be responsible for the IFN-γ production in CBMC upon EBV encounter *in vitro* (Wilson and Morgan, 2002). The frequency of NK cells in CBMC and PBMC is similar, but NK cells in CBMC have an immature function compared with NK cells in PBMC (Nomura *et al*., 2001). To determine if the immune activation/maturation deficiencies in IL-12 and IFN-γ production indeed enable *BZLF-1* mRNA expression in CBMC cultures, we added rIL-12 and rIFN-γ to the CBMC

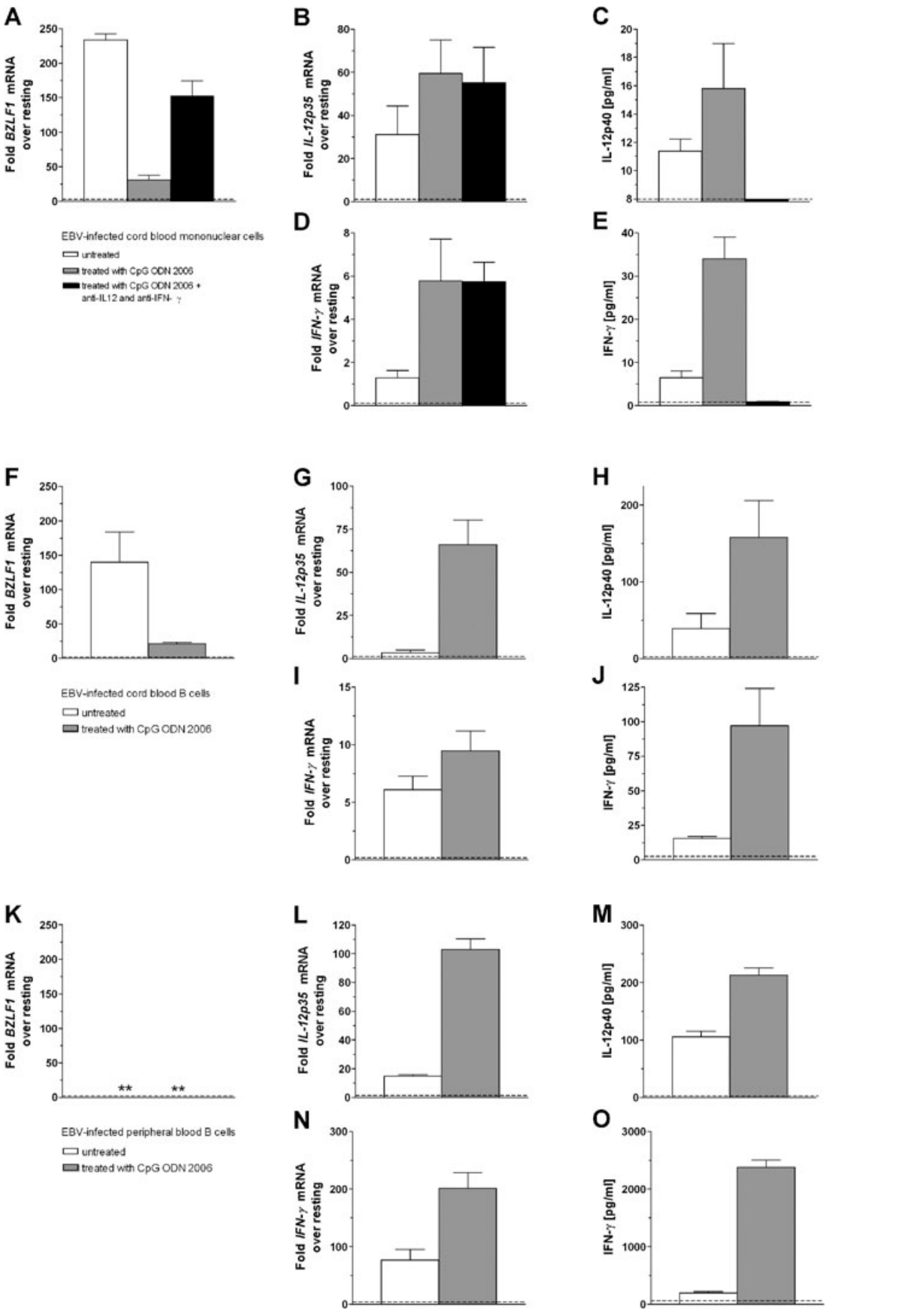

Fig. 7. *BZLF-1* mRNA expression in CpG ODN 2006-treated CBMC infected *ex vivo* with EBV is dependent on IL-12 and IFN-γ expressed in B lymphocytes.
A. mRNA expression of *BZLF-1* in CBMC infected *ex vivo* with EBV and treated or not with CpG ODN 2006 and with or without anti-IL12 plus anti-IFN-γ blocking antibodies.
B. mRNA expression of *IL-12p35* in CBMC infected *ex vivo* with EBV and treated or not with CpG ODN 2006 and with or without anti-IL12 plus anti-IFN-γ antibodies.
C. IL-12p40 in supernatants of CBMC infected *ex vivo* with EBV and treated or not with CpG ODN 2006 and with or without anti-IL12 plus anti-IFN-γ antibodies.
D. mRNA expression of *IFN-γ* in CBMC infected *ex vivo* with EBV and treated or not with CpG ODN 2006 and with or without anti-IL12 plus anti-IFN-γ antibodies.
E. IFN-γ in supernatants of CBMC infected *ex vivo* with EBV and treated or not with CpG ODN 2006 and with or without anti-IL12 plus anti-IFN-γ antibodies.
F. mRNA expression of *BZLF-1* in $CD19^+$ B cells isolated from cord blood infected *ex vivo* with EBV.
G. mRNA expression of *IL-12p35* in $CD19^+$ B cells isolated from cord blood infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
H. IL-12p40 in supernatants of $CD19^+$ B cells isolated from cord blood infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
I. mRNA expression of *IFN-γ* in $CD19^+$ B cells isolated from cord blood infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
J. IFN-γ in supernatants of $CD19^+$ B cells isolated from cord blood infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
K. mRNA expression of *BZLF-1* in $CD19^+$ B cells isolated from peripheral blood infected *ex vivo* with EBV.
L. mRNA expression of *IL-12p35* in $CD19^+$ B cells isolated from peripheral blood infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
M. IL-12p40 in supernatants of $CD19^+$ B cells isolated from peripheral blood infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
N. mRNA expression of *IFN-γ* in $CD19^+$ B cells isolated from peripheral blood infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
O. IFN-γ in supernatants of $CD19^+$ B cells isolated from peripheral blood infected *ex vivo* with EBV and treated or not with CpG ODN 2006.
CpG ODN 2006 (1 μM) was added at 0 and 90 h to the EBV-containing culture medium of 2×10^6 CBMC. Anti-IL-12 and anti-IFN-γ antibodies were given to the cultures 1 h before stimulation with CpG ODN 2006. Cells were collected at 96 h. RNA was extracted from two cell pellets per condition, treated with DNase, and reverse-transcribed into cDNA with an oligo-dT15 primer. mRNA expression was measured in duplicate by real-time PCR. Results are means ± SD of fold induction over resting normalized to the housekeeping gene HMBS. The dashed lines indicate the lower limit of detection. One representative experiment of two is shown.

cultures infected *ex vivo* with EBV. Indeed, *BZLF-1* mRNA expression in CBMC decreased significantly, albeit not completely, with rIL-12 and rIFN-γ. The incomplete suppression of *BZLF-1* mRNA expression may be explained by immature cytokine receptor signalling pathways in CBMC (Marodi, 2002) or the need of additional stimuli operative in the innate immune responses (Medzhitov, 2001). Thus, activation of the immune system results in efficient suppression of the initiation of lytic EBV infection.

The anti-inflammatory cytokine TGF-β induces lytic infection in EBV-transformed CBMC-derived cell lines (Liang *et al.*, 2002). Thus, we explored the possibility that

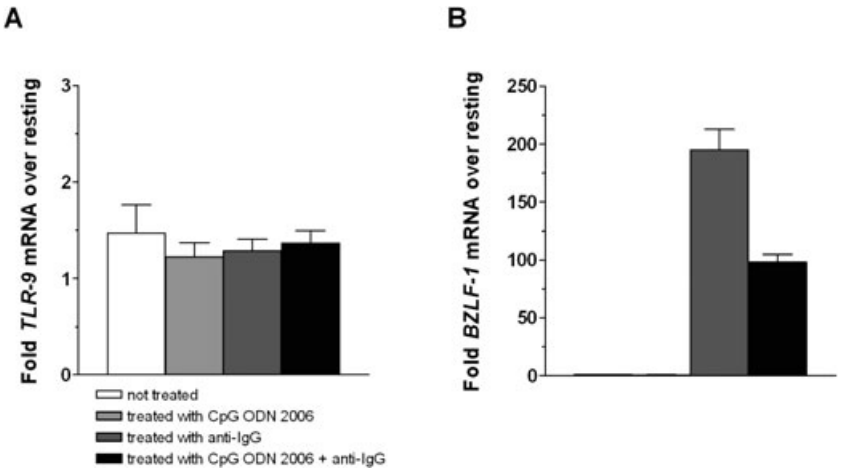

Fig. 8. CpG ODN 2006 suppresses induction of *BZLF-1* mRNA expression in Akata Burkitt lymphoma cells provoked to switch to lytic EBV infection.
A. Expression of *TLR-9* mRNA before and after stimulation with CpG ODN 2006, anti-IgG, or both.
B. Expression of *BZLF-1* mRNA before and after stimulation with CpG ODN 2006, anti-IgG, or both.
Akata cells (1.0×10^6 each) were seeded and treated with or without CpG ODN 2006 (0.5 μM) After 6 h 0.1 μg μl^{-1} polyclonal rabbit anti-human IgG was added to the cultures. Cells were collected at 6 h after anti-IgG treatment. RNA was extracted from one cell pellet per condition, treated with DNase, and reverse-transcribed into cDNA with an oligo-dT15 primer. mRNA expression was measured in duplicate by real-time PCR. Results are means ± SD of fold induction over resting normalized to the housekeeping gene HMBS from three independent experiments.

the lower levels of IL-12, IFN-γ and IL-2 in CBMC than in PBMC were coupled to higher mRNA levels of *TGF-β*. However, *TGF-β* mRNA expression was lower in CBMC than in PBMC irrespective of *ex vivo* EBV infection, making a contribution of TGF-β to *BZLF-1* expression in CBMC highly unlikely. Another possible reason for the lower IL-12, IFN-γ and IL-2 levels in CBMC than in PBMC could have been increased levels of the anti-inflammatory cytokine IL-10 (Wang *et al*., 1994). The lower mRNA levels of *IL-10* in CBMC in response to EBV infection compared with in PBMC, however, argued against IL-10 being responsible for the lower levels of proinflammatory cytokines in CBMC. Notably, adding or blocking IL-10 had no effect on lytic EBV gene expression patterns.

We wanted to verify our observation that activation of the immune system results in suppression of *BZLF-1* by activating the innate immune response triggering TLR-9. Indeed, triggering of innate immunity via TLR-9 with CpG ODN 2006 resulted in suppression of *BZLF-1* but not latent EBV gene mRNA expression in acutely *ex vivo* EBV-infected CBMC. Notably, triggering TLR-9 resulted in higher transformation rates of B cells infected *ex vivo* with EBV (Traggiai *et al*., 2004), but the effect of stimulating TLR-9 of B cells on EBV gene expression was not investigated. Thus, the molecular mechanism(s) resulting in the more efficient transformation rate of B cells when triggering TLR9 may be due to reduction of initiation of lytic EBV infection and thereby reinforce maintenance of EBV latency.

Our results seem to be in conflict with the findings of Liu *et al*. (2005) and Lim *et al*. (2007). Liu *et al*. (2005) reported that truncated thioredoxin (Trx80) inhibits B cell growth in EBV infected CBMC through T cells activated by monocyte derived IL-12. They assessed B cell transformation by EBV by measuring the thymidine incorporation on the 12th day. Patterns of EBV latent and lytic gene expression were not investigated. In contrast, our experiments focusing on acute *ex vivo* EBV infection were limited to 7 days. Through the use of isolated B cells in selected experiments we showed that IL-12 derived from B cells mediated suppression of lytic EBV. We did not assess B cell transformation. Thus, the results of these two studies cannot be directly compared due to the different experimental settings used; they are not mutually contradictory. Future experiments may resolve this enigma. Furthermore, Lim *et al*. (2007), reported that human plasmacytoid DCs regulated immune responses to EBV in humanized NOD-SCID mice resulting in delayed EBV-related mortality. From indirect proof using an inhibitor for triggering TLR-9 they concluded that TLR-9 in part mediated activation of plasmacytoid DCs resulting in anti-EBV-active $CD3^+$ T cells. In this study, PBMC from EBV-seropositive donors were used; thus, the protective effect observed is most likely due to the boosting effect of an adaptive EBV-specific cellular immune response.

Next, we addressed the question whether TLR-9 triggering affects on EBV in chronically infected cells. Chronically EBV-infected cells express latent EBV genes and only very rarely lytic EBV genes. To assess the effects of triggering of TLR-9 on lytic EBV in chronically EBV-infected cells, we used Akata Burkitt lymphoma cells, which undergo lytic EBV infection upon anti-IgG stimulation (Kieff and Rickinson, 2001). Similarly to *ex vivo* acutely infected B cells, TLR-9 triggering suppresses anti-IgG-induced *BZLF-1* expression in Akata cells. This result also indicates that triggering TLR-9 directly affects the EBV gene expression pattern and is not a consequence of indirect effects due to stimulation of other cellular subsets. Of note in this context, *Plasmodium falciparum* malaria pigment hemozoin also stimulates TLR-9 (Coban *et al*., 2005). Children in areas endemic for both EBV-positive Burkitt lymphoma and malaria are dually infected with EBV and malaria very early in life (Rochford *et al*., 2005). We show that suppression of lytic EBV via TLRs on and in B cells is specifically linked to triggering of TLR-9 and that suppression of lytic EBV occurs following direct triggering of TLR-9 in B cells. Thus, repeated activation of the innate immunity via TLR-9 (e.g. due to chronic malaria infection) may foster the propagation of latently EBV-infected cells by suppressing lytic EBV infection and thus development of Burkitt lymphoma.

We and others have documented increased plasma EBV DNA levels in patients with IM or EBV-associated lymphoproliferative diseases in immunocompetent and immunodeficient patients (Berger *et al*., 2001; Ryan *et al*., 2004) as well as in individuals with malaria (Moormann *et al*., 2005; Donati *et al*., 2006). Plasma EBV DNA is sensitive to DNase; this indicates that it is not encapsidated and does not originate from lytic infection but rather from dying latently infected cells (Ryan *et al*., 2004; Donati *et al*., 2006). Moorman *et al*. also found elevated EBV DNA blood levels in children with malaria and suggested as likely reasons an increased frequency of latently EBV-infected cells, indirectly due to polyclonal B cell activation or due to suppression of EBV-specific immunity, and that recurrent malaria infections affect either the establishment or maintenance of EBV latency (Moormann *et al*., 2005). Notably, no *BZLF-1* transcription is found in PBMC from IM patients (Tierney *et al*., 1994) with high serum levels of IL-12, IFN-γ and IL-2 (Corsi *et al*., 2004). This is in line with our findings showing IL-12- and IFN-γ-mediated suppression of *BZLF-1* expression. Our findings are further supported by the observations that IFN-γ blocks gammaherpesvirus reactivation from latency (Steed *et al*., 2006) and nuclear factor κB, activated downstream of TLR-9, inhibits gammaherpesvirus lytic replication (Brown *et al*., 2003). Thus, we

hypothesize that in states of increased immune activation propagation of EBV is due to promotion of latent rather than of lytic EBV infection. Because control of EBV infection may substantially differ between tissue compartments (Hislop *et al*., 2005; Donati *et al*., 2006), in states of immune activation or cellular immune compromise lytic EBV infection may be confined to tissues at mucosal surfaces with excretion of EBV particles.

Experimental procedures

Isolation of mononuclear cells and cell culture

Cord blood mononuclear cells and PBMC from healthy adult EBV-seronegative donors were obtained from heparinized blood by Ficoll-Hypaque (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland) gradient centrifugation. Cells were washed with phosphate-buffered saline (Gibco, Invitrogen Life Sciences, Basel, Switzerland). The EBV-producing cell lines B95.8 (Miller and Lipman, 1973) and B95.8EBfaV-GFP (Speck and Longnecker, 1999), CBMC, PBMC and Akata (Takada, 1984) cells were cultured in RPMI 1640 supplemented with Hepes buffer, L-glutamine, 10% fetal bovine serum, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin sulphate (medium and supplements from Gibco). Informed consent was obtained from subjects or parents before the study. The institutional ethics committee approved the collection and use of clinical material.

Isolation of B cells from CBMC and PBMC

B cells were isolated from CBMC or PBMC by the use of magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Purity of isolated B cells was determined by flow cytometry using anti-human CD19, and anti-human CD3 antibodies for the detection of B cells and eventually remaining T cells. The purity of each separation was above 97%.

Epstein-Barr virus infections ex vivo

After resting overnight, CBMC or PBMC (1×10^7 cells) were infected with supernatants from B95.8 cells or B95.8EBfaV-GFP (1×10^6 ml^{-1}) harvested on day 4 after splitting and filtered using a 0.45 μm sterile filter (Millipore, Cork, Ireland). The cell-free supernatants contained approximately 7 log_{10} EBV copies ml^{-1}, as evaluated by real-time PCR for EBV DNA (Berger *et al*., 2001). Infections were performed as described (Tosato, 1991). Briefly, cells were centrifuged, resuspended in 2.5 ml of RPMI and 2.5 ml of B95.8 supernatant, and incubated in 50 ml conical Falcon tubes (BD Biosciences, Basel, Switzerland) at 37°C in a water bath for 2 h. Subsequently, 5 ml of RPMI 1640 were added, and 1 ml aliquots (1×10^6 cells ml^{-1}) were seeded into 24-well plates (BD Biosciences). Cell pellets were centrifuged at 300 *g*, frozen on dry ice, and stored at −80°C.

Assessment of EBV and cytokine gene transcription

RNA extractions were performed with the RNA Easy Extraction kit (Qiagen, Basel, Switzerland), according to the supplier's instructions. RNA was treated with DNase [DNAfree; Ambion (Europe), Huntington, Cambridgeshire, UK] for removal of residual DNA. RNA (1 μg) was reverse transcribed in a total volume of 20 μl with oligo-dT15 primer (Microsynth, Balgach, Switzerland) using Omniscript Reverse Transcription kit (Qiagen). RNase inhibitor (10 units) (RNasin plus, Promega, Catalys AG, Wallisellen, Switzerland) was added to each 20 μl reaction. Real-time PCR (TaqMan) for human *IL-2, IL-12 p35, IFN-γ, IL-1β, IL-6, IL-8, IL-10, TGF-β, TNF-α* genes, EBV nuclear antigen *(EBNA)-1, EBNA-2, latent membrane protein (LMP)-1, LMP-2, BamHI Z fragment (BZLF)*-1, *glycoprotein (gp) 85* (C. Berger, *et al.* submitted), and the housekeeping gene, *hydroxymethylbilane synthase (HMBS)*, were performed according to the supplier's instructions (Applied Biosystems, Foster City, CA, USA) and as described (Bonanomi *et al*., 2003). The assays were cDNA specific: either the forward or reverse primer or the probe was designed to span exon-exon junctions. Specificity (DNA/cDNA) was tested using RNA before and after DNase treatment and cDNA with or without prior DNase treatment. The assay for human *IL-10* is highly specific and does not detect viral *IL-10* (data not shown). All reactions were performed in duplicate. Each 15 μl reaction contained a mix of the 2× ABI-TaqMan Master Mix (Applied Biosystems), primers (Microsynth) at 300 nM each, the probe (Biosearch Technologies, Novato, CA, USA) at 200 nM, and 1 μl of cDNA template. Ct values obtained for HMBS were used for normalization. Both positive (amplified cDNA sequences of the selected cytokines or EBV genes tested) and negative controls (no template) were included on every plate.

Immunofluorescence

Cord blood mononuclear cells were washed in PBS, transferred to coated slides in a Cytospin 3 centrifuge (Shandon, Histocom, Zug, Switzerland), air dried, fixed with acetone at 4°C, and stored at −20°C. After thawing and before staining, the cells were blocked with 5% goat serum in PBS, incubated with the anti-BZLF-1 antibody (1:40; Clone BZ.1, DakoCytomation, Zug, Switzerland), followed by the secondary goat anti-mouse IgG antibody labelled with the green fluorescent Alexa Fluor 488 dye (Molecular Probes-Invitrogen, Basel, Switzerland). Nuclei were stained with 4,6 diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Analysis was carried out with the Zeiss AXIOSKOP 2 Mot Plus microscope, the Plan Neofluar 20 ×/0.50 Ph2 objective, the Fluoarc Lamp, the AxioCam MR and the AxioVision 3.1 software (all from Carl Zeiss AG, Oberkochen, Germany). Adobe Photoshop 6.0 was used to magnify the region of interest.

Flowcytometric analyses to determine T-cell activation or fractions of EBV-infected B cells

The cell pellets were resuspended and washed in staining buffer (PBS with 5% FBS and 0.1% sodium azide but without Ca^{+2} or Mg^{+2}). Cells were double stained with an FITC-labelled and a PE-labelled mouse anti-human monoclonal antibody (all from BD Biosciences, if not stated otherwise) at 4°C in the dark for 30 min. As isotype controls, FITC-conjugated anti-mouse IgG_1 (FITC-IgG_1) with PE-conjugated anti-mouse IgG_1 (PE-IgG_1) and FITC-IgG_1 with PE-HLA-ABC were used. Activated T cells were evaluated with FITC-anti-HLA-DR and either PE-anti-CD4 or

PE-anti-CD8. B cells were evaluated with PE-anti-CD19 and Cy5-anti-CD5. Detection of the late lytic EBV glycoprotein gp350/220 was performed using a FITC-labelled anti-EBV gp350/220 antibody (Milan Analytica, La Roche, Switzerland).The samples were analysed using a FACSCalibur (BD Biosciences) equipped with 488 nm and 635 nm lasers for double colour analysis. Events (10 000 per lymphocyte gate) were recorded and analysed with the Cell Quest software (BD Biosciences).

Assessment of cytokine levels

Samples were analysed using multiplex bead analysis that uses microspheres as the solid support for immunoassays (Chen *et al.*, 1999). Cytokine levels were measured according to the manufacturer's instructions (Upstate Biotechnology UK, Buckingham, UK).

Stimulation of CBMC with rIFN-γ, rIL-12, or IL-10 and inhibition of IL-12 or IFN-γ by addition of anti-IL-12, anti-IFN-γ, or IL-10 antibodies

Cord blood mononuclear cells (1×10^6 ml^{-1}) were infected with EBV as described above, but with or without addition of 20 ng ml^{-1} rIL-12, or 10 ng ml^{-1} rIFN-γ, or both (both from R&D Systems, Abingdon, UK), or 1, 10, or 100 pg ml^{-1} IL 10 (Peprotech EC, London, UK). RIL-12, rIFN-γ, or both, or IL-10 were added in 24 h intervals to the cells. CBMC or PBMC (1×10^6 ml^{-1}) were infected with EBV with or without 100 ng anti-IL-12, 1 μg anti-IFN-γ antibodies (both from R&D Systems), or anti-IL-10 antibodies (Biolegend, San Diego, USA).

Stimulation of CBMC, PBMC, or Akata cells with ligands to TLRs

Cells (3 or 5×10^6 cells ml^{-1}) were left uninfected or infected with EBV and were stimulated with TLR ligands added at 0 h and 90 h. Concentrations of TLR ligands were 10 μg ml^{-1} for peptidoglycan (TLR1/2), 20 μg ml^{-1} for lipopolysaccharide (TLR4), 3 μM for R-848 (TLR7/8) and 0.5 μM for CpG ODN 2006 (TLR9) (InvivoGen, San Diego, CA, USA). The cells were kept in culture for a total of 96 h.

Initiation of lytic EBV infection in Akata Burkitt lymphoma cells

Akata cells were split to a concentration of 1×10^6 cells ml^{-1} 24 h before stimulation. Cells (1×10^6 ml^{-1}) were stimulated with 0.1 μg μl^{-1} polyclonal rabbit anti-human IgG (Dako, Zug, Switzerland) and suspended in fresh RPMI 1640. After 6 h, stimulated cells were collected for RNA isolation.

Statistical analyses

The Mann–Whitney *U*-test (two-tailed) was used for comparison of differences between groups. The level of statistical significance was set at $P < 0.05$.

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IMMUNOLOGY ORIGINAL ARTICLE

Plasma cell toll-like receptor (TLR) expression differs from that of B cells, and plasma cell TLR triggering enhances immunoglobulin production

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Summary

Toll-like receptors (TLRs) are key receptors of the innate immune system and show cell subset-specific expression. We investigated the messenger RNA (mRNA) expression of *TLR* genes in human haematopoietic stem cells (HSC), in naïve B cells, in memory B cells, in plasma cells from palatine tonsils and in plasma cells from peripheral blood. HSC and plasma cells showed unrestricted expression of *TLR1–TLR9*, in contrast to B cells which lacked *TLR3*, *TLR4* and *TLR8* but expressed mRNA of all other *TLR*s. We demonstrated, for the first time, that TLR triggering of terminally differentiated plasma cells augments immunoglobulin production. Thus, boosting the immediate antibody response by plasma cells upon pathogen recognition may point to a novel role of TLRs.

Keywords: B-cell subpopulations; immunoglobulin production; mRNA expression; plasma cells; toll-like receptors

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Introduction

Toll-like receptors (TLRs) are key recognition structures of the innate immunity.[1] They trigger antimicrobial responses by conserved pathogen-associated molecular patterns. The signalling cascade culminates, among others, in the activation of nuclear factor-κB (NF-κB), which results in the expression of pro-inflammatory cytokines that are critical for the innate as well as for the adaptive immune responses.[1] Furthermore, TLRs play a role in autophagy,[2] haematopoiesis[3] and neutrophil activation.[4] For each of the 10 known human TLRs (TLR1–TLR10), at least one distinct ligand has been identified, except for TLR10.[1]

Cell subpopulations exhibit specific TLR expression patterns,[5,6] indicating that TLR expression is tailored to distinct cellular functions. Furthermore, the TLR expression pattern depends on the developmental stage, as exemplified by the developmental-dependent degree of *TLR1–TLR5* expression in dendritic cells[7] and of *TLR9* in B cells.[8] Delineation of the expression of TLRs during human B-cell development is so far incomplete.[9] Data on TLR expression in human B cells at their maturation stages and in terminally differentiated plasma cells from the same donors and from the same secondary lymphatic organ where differentiation actually takes place (e.g. tonsils), have not yet been presented, and such data form the basis for functional studies.

Here, we hypothesized that the expression and function of TLRs are tailored to stages of B-cell development and differentiation. Therefore, we investigated the quantitative expression of *TLR1–TLR10* in haematopoietic stem cells

(HSC) as well as in naïve B cells, memory B cells and plasma cells from the same lymphoid tissue. Furthermore, we addressed the as-yet uninvestigated issue of whether TLR triggering affects plasma cell function, and we showed that TLR triggering increases the production and secretion of immunoglobulin from plasma cells.

Materials and methods

Cells and subpopulations

Cells were isolated from cord blood or tonsils, as described previously.[10–12] The study was approved by the local ethics committee, and written informed consent was obtained for all tissue obtained. Cord blood HSC, B cells and plasma cells were isolated using CD34 microbeads, the B-cell isolation kit II and CD138 microbeads, respectively, according to the instructions of the manufacturer (Miltenyi Biotech, Bergisch Gladbach, Germany). Further separation of B cells into naïve and memory B cells was performed using the naïve-B-cell isolation kit (Miltenyi Biotech) or CD27 microbeads (Miltenyi Biotech).[12] Isolated cell populations used for experiments were always > 95% pure, as determined by flow cytometry.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (PCR) was performed for *TLR9* and the housekeeping gene *hydroxymethylbilane-synthase* (*HMBS*), as described previously.[10,12] *TLR10* was analyzed using primer/probe on demand (Hs01935337_s1, Assay-on-demand gene expression product; Applied Biosystems, Foster City, CA). SYBR Green primers for *HMBS* and *TLR1–TLR8* were as described previously.[13]

Flow cytometry

Flow cytometry using fluorochrome-conjugated monoclonal antibodies to human CD34, CD19, CD27, CD138, IgM or IgG (BD Biosciences, Basel, Switzerland) was executed on a Cytomics FC500 instrument (Beckman Coulter, Nyon, Switzerland); data were analyzed using FLOWJO software (Treestar, Ashland, OR).

Intracellular immunoglobulin staining and enzyme-linked immunosorbent assay

Tonsillar plasma cells were either untreated or were stimulated with 10 μg/ml of peptidoglycan (TLR1/2 ligand; Fluka, Buchs, Switzerland), 1 μg/ml of poly(I:C) (TLR3 ligand; InvivoGen, San Diego, CA), 10 ng/ml of lipopolysaccharide (LPS) (TLR4 ligand; Sigma-Aldrich, Buchs, Switzerland), 10 ng/ml of flagellin (TLR5 ligand; InvivoGen), 3 μM R-848 (TLR7/8 ligand; InvivoGen), or 2 μM cytosine–phosphate–guanosine (CpG) oligonucleotide (ODN) 2006 (TLR9 ligand; Eurogentec, Köln, Germany). Seventy-two hours after stimulation, cells were harvested, fixed, permeabilized and stained. Intracellular staining of IgM and IgG on plasma cells was performed using fluorochrome-conjugated monoclonal antibodies and the BD Cytofix/Cytoperm kit (both from BD Biosciences), according to the manufacturer's instructions.

The total amount of secreted immunoglobulin was determined using an in-house enzyme-linked immunosorbent assay: briefly, 96-well microtitre plates were coated with 10 μg/ml of Protein G (Calbiochem, Dietlikon, Switzerland) diluted in a carbonate–bicarbonate buffer (pH 9·6) and stored overnight at room temperature in a humid chamber. The plates were washed four times with phosphate-buffered saline (PBS) and incubated for 1 hr at room temperature with 200 μl per well of 3% bovine serum albumin in PBS. After discarding the blocking buffer, 50 μl of supernatant of the plasma cell samples, or serial dilutions of human immunoglobulin (NIBSC, Hertfordshire, UK) as a reference, were added to each well and allowed to react for 30 min at 37°. After three washing steps, peroxidase-labelled sheep anti-human immunoglobulin (Millipore, Munich, Germany) was incubated for 30 min at 37°. After three washing steps, 100 μl of 3,3′,5,5′-tetramethyl-benzidine substrate (Mabtech, Hamburg, Germany) was added and incubated for 30 min at 37° in the dark. The reactions were stopped by the addition of 50 μl of 1 M citrate. The absorbance was determined photometrically at 450 nm, with 620 nm as the reference filter.

Results

Pattern of TLR mRNA expression changes during B-cell development

We aimed to assess the TLR expression patterns of distinct human B-cell subpopulations, including HSC and naïve and memory B cells, as well as plasma cells (Fig. S1). TLR protein levels may correlate with mRNA expression for cell-surface TLRs[14,15] but in our hands commercially available TLR antibodies failed to reproduce previously described results on the subcellular localization of TLRs (data not shown). Thus, we quantified TLR expression using real-time PCR to TLR mRNA. Indeed, TLR expression patterns display notable changes during B-cell development. HSC and tonsillar plasma cells expressed all *TLRs* with the exception of *TLR10* (Fig. 1). In contrast to HSC and plasma cells, we found a complete absence of *TLR3*, *TLR4* and *TLR8* expression in naïve and memory B cells, which is in agreement with published data,[5,6,16] while the other *TLR*s were expressed to varying degrees (Fig. 1). Next, we investigated whether circulating plasma cells differ in their *TLR* expression

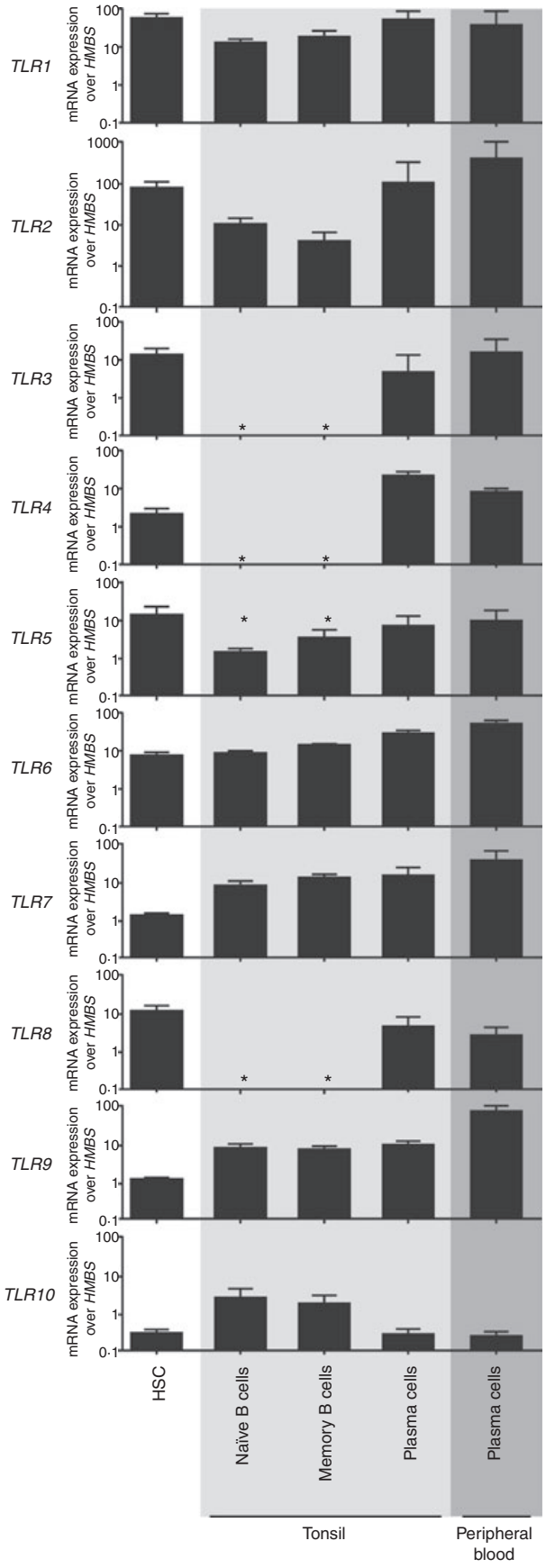

Figure 1. Messenger RNA (mRNA) expression levels of toll-like receptor (*TLR)1–TLR10* show distinct regulation patterns during B-cell development. mRNA expression profiling is shown of *TLR1–TLR10* in haematopoietic stem cells (HSC) isolated from cord blood, in naïve B cells, in memory B cells, in terminally differentiated plasma cells from tonsils and in terminally differentiated plasma cells from peripheral blood. HSC and plasma cells were isolated by positive selection using CD34 microbeads and CD138 microbeads, respectively. Naïve B cells and memory B cells were isolated using the naïve B-cell isolation kit and a combination of the B-cell isolation kit II and CD27 microbeads, respectively. Expression of *TLR1–TLR10* and of the housekeeping gene *hydroxymethylbilane-synthase* (*HMBS*) mRNA was monitored by quantitative real-time polymerase chain reaction (PCR). Results shown are the means ± standard deviation (SD) of three biological replicates of one out of three representative experiments. *Denotes not detectable.

from their tonsillar counterparts to exclude the possibility plasma cells exhibit distinct *TLR* expression patterns after exiting secondary lymphoid organs. We found that plasma cells from the peripheral blood exhibited the same expression pattern as tonsillar plasma cells but that expression of TLR9 was around 10-fold higher (Fig. 1). The cell subset-specific TLR expression observed here suggests that HSC and periphery terminally differentiated plasma cells need the expression of *TLR1–TLR9*, whereas B cells from secondary lymphoid organs need expression of *TLR1*, *TLR2*, *TLR5–TLR7*, *TLR9* and *TLR10*.

Engagement of TLRs on terminally differentiated plasma cells increases immunoglobulin production and secretion

Based on the constitutively high *TLR1–TLR9* expression by plasma cells (i.e. of the TLRs for which ligands are known), we wondered whether TLR triggering affects the function of these cells. Indeed, plasma cell TLR triggering resulted in significantly increased production of intracellular IgM following triggering of TLR1/2 (Fig. 2a,c) and of IgG following triggering of TLR3 or TLR9 (Fig. 2b,d) as well as secretion of total immunoglobulin following triggering of TLR1–TLR3 and TLR5 (Fig. 2e), when compared with freshly *ex vivo* isolated plasma cells.

Next, we evaluated whether the increased TLR9 expression by plasma cells from peripheral blood compared with that from tonsils influences immunoglobulin production upon TLR triggering (Fig. 3). We observed a significant increase of intracellular IgM production following triggering of TLR1–TLR4 (Fig. 3a,c), and a significant increase of intracellular IgG production following triggering of TLR7/8 (Fig. 3b,d), when compared with freshly isolated plasma cells *ex vivo*.

This increased immunoglobulin production/secretion suggests that terminally differentiated plasma cell TLRs

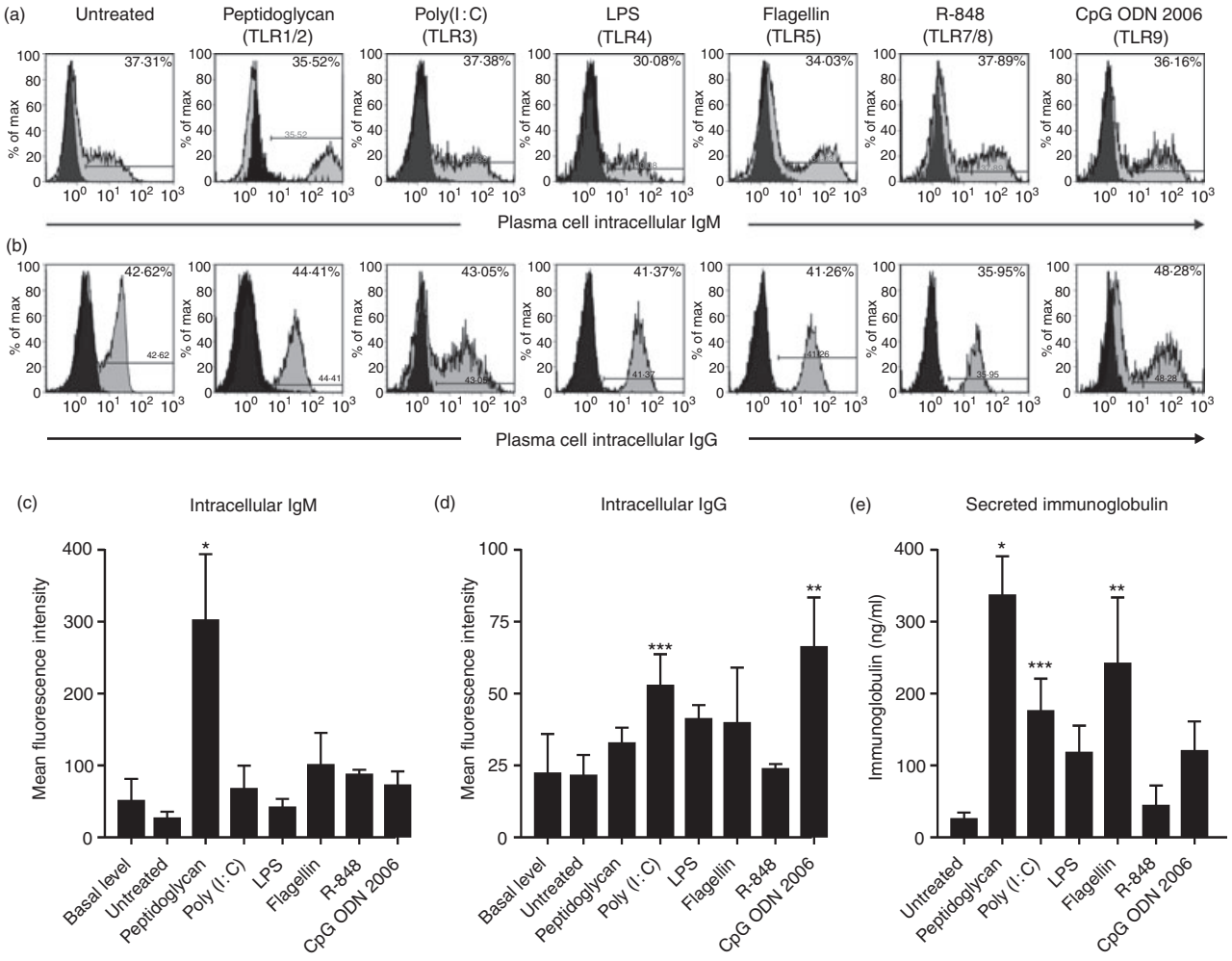

Figure 2. Triggering of toll-like receptors (TLRs) on plasma cells isolated from tonsils induces increased production of immunoglobulin. Intracellular (a) IgM and (b) IgG expression in plasma cells, mean fluorescence intensity of (c) IgM and (d) IgG in plasma cells and (e) secretion of immunoglobulin by plasma cells isolated from tonsils. Plasma cells were isolated from palatine tonsils by positive selection using CD138 microbeads. The viability of plasma cells after 72 hr in culture was always above 75%. Black histograms indicate isotype-control staining. The results shown are the means ± standard deviation of three biological replicates of one out of three representative experiments. $^{*}P < 0{\cdot}001$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}05$, by Kruskall–Wallis and Dunn's multicomparison test. CpG ODN, cytosine–phosphate–guanosine oligonucleotide 2006; LPS, lipopolysaccharide.

have functions beyond mere innate immunity pathogen sensing and thus may be critical in regulating the strength of adaptive antibody-mediated immune responses. The omnipresent *TLR1–TLR9* mRNA expression by plasma cells seems to contrast the selective, strong responses to TLR1–TLR3, TLR5, or TLR9 triggering in tonsillar plasma cells and to TLR1–TLR4 and TLR7/8 triggering in peripheral blood plasma cells. One possible explanation for this could be that different TLRs drive different classes of antibodies. Nevertheless, the selective responses observed may depend on the specific adaptive immunity repertoire of the plasma cell donor, as indicated by the divergent results obtained for TLR3 and TLR7/8 triggering of tonsillar versus peripheral blood plasma cells, which were obtained from different donors.

Discussion

In the present study we aimed to assess the TLR expression patterns in human B cells (including HSC, naïve and memory B cells, as well as plasma cells) at distinct developmental stages and to analyze the impact of plasma cell TLR engagement. Indeed, we demonstrated that TLR expression patterns display notable changes during peripheral B-cell development, that plasma cells exhibit TLR expression distinct from naïve and memory B cells but similar to HSC, and that engagement of plasma cell TLRs results in augmented immunoglobulin production and secretion by these cells. These results suggest that TLR expression is tailored to cellular developmental stage function.

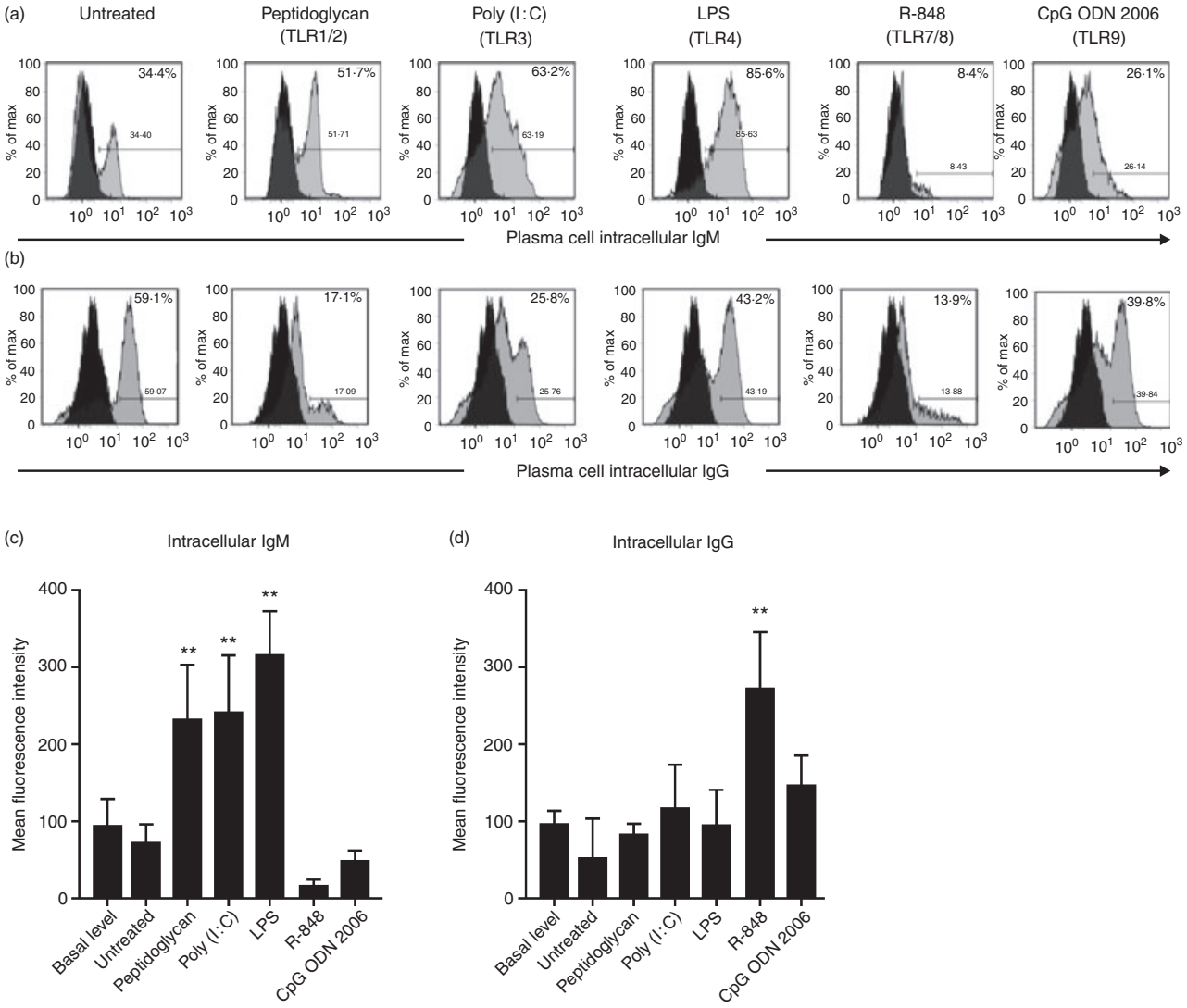

Figure 3. Triggering of toll-like receptors (TLRs) on plasma cells isolated from peripheral blood induces increased production of immunoglobulin. Intracellular (a) IgM and (b) IgG expression, and mean fluorescence intensity of (c) IgM and (d) IgG, in plasma cells isolated from peripheral blood. Plasma cells were isolated from peripheral blood by positive selection using CD138 microbeads. The viability of plasma cells after 72 hr in culture was always above 75%. Black histograms indicate isotype-control staining. The results shown are the means ± standard deviation of three biological replicates of one out of three representative experiments. $^{**}P < 0{\cdot}01$, by Kruskall–Wallis and Dunn's multicomparison test. CpG ODN, cytosine–phosphate–guanosine oligonucleotide 2006; LPS, lipopolysaccharide.

In agreement with published data,[5,6,16] we found a complete absence of *TLR*3, *TLR4* and *TLR8* expression in naïve and memory B cells, while varying levels of the other *TLR*s were expressed in those cells. We complemented the characterization of the TLR profile in the B-cell lineage by examining terminally differentiated plasma cells: we found that plasma cells expressed all TLRs, with the exception of TLR10 which is very weakly expressed. By contrast, *TLR10* was expressed very strongly in naïve and memory B cells. Strikingly, HSC expressed virtually no *TLR10*. Lack of acceptable specificity of antibodies to TLRs (data not shown) precluded analysis at the protein level. Triggering TLRs resulted in increased production of IgM and IgG as well as in the secretion of soluble immunoglobulin by plasma cells, which also speaks in favour of the functional integrity of TLRs and their immunological significance. This increased immunoglobulin production/secretion points to a novel role of TLRs: TLRs are known for their role in the innate immune response as well as for linking the innate and adaptive immune responses by enhancing cellular activation, up-regulation of the major histocompatibility complex (MHC) class molecules and release of cytokines; the fact that TLR agonists directly engage in the generation and strength of antibody production uncovers a novel role that may have therapeutic application (e.g. boosting

an antibody response). Noteworthy, we observed preferential production of IgM following triggering of TLR1/2 and of IgG following triggering of TLR3 or TLR9, as well as a preferential secretion of immunoglobulin following triggering of TLR1/2, TLR3, or TLR5 by terminally differentiated plasma cells isolated from tonsils. By contrast, we observed preferential production of IgM following triggering of TLR1–TLR4, and of IgG following triggering of TLR7/8, by terminally differentiated plasma cells isolated from peripheral blood. This may be a result either of preferential recognition and specialized function of TLRs in terminally differentiated plasma cells in response to distinct conserved motifs or because of the preferential isotype immunoglobulin responses of the plasma cells we purified. The latter is dependent on the donor's immunity repertoire. Previous analyses of TLR function in B-cell lineages have shown that naïve and memory B cells respond to TLR agonists by sustaining or increasing their TLR expression or by inducing the expression of pro-inflammatory cytokines such as interleukin-8 (IL-8).[5,6,16] Thus, the data we report outline a higher complexity of TLR function in B-cell immunology when expanded to terminal plasma cell differentiation.

Our observation of TLR expression by plasma cells from tonsils or peripheral blood is in contrast to that reported for plasma cells from the bone marrow, which shows largely the absence of TLR expression.[17] It is generally agreed that the bone marrow is the primary site of long-lived plasma cells which are responsible for at least some persistent antibodies, and that occur independently of memory B cells and antigen.[18] Thus, given their continuous antibody production, long-lived plasma cells in the bone marrow may not need to be boosted via TLR triggering and therefore TLR expression by bone marrow plasma cells may be dispensable. Intriguingly, we found that peripheral blood plasma cells expressed TLR9 at approximately 10-fold higher levels than their tonsillar counterparts. Nevertheless, triggering of TLR9 in peripheral blood plasma cells did not result in increased intracellular IgG production, as observed in tonsillar plasma cells. Thus, the level of TLR9 expression seems not to correlate to the level of augmented immunoglobulin production upon triggering. A possible explanation for this may be that only a small portion of TLR9 is cleaved to the active form.[19]

The cell subset-specific TLR expression observed here suggests that HSC and periphery plasma cells need the expression of *TLR1–TLR9*, whereas B cells from secondary lymphoid organs need the expression of *TLR1*, *TLR2*, *TLR5–TLR7*, *TLR9* and *TLR10*. We speculate that the distinct TLR profile of HSC is critical for their differentiation towards a specific cell subset and dependent upon the encountered pathogen recognition pattern to optimize pathogen-driven immune responses. Indeed, recent work in mice[3] and humans[20] has demonstrated that TLR signals bias HSC towards myelopoiesis directly by replacing endogenous cytokines normally required for the survival, proliferation and development of haematopoietic progenitors. In naïve and memory B cells, TLR triggering seems to be restricted to sustaining or increasing their TLR expression or inducing the expression of pro-inflammatory cytokines such as IL-8.[5,6,16] Finally, as we show, peripheral plasma cell TLR engagement augments immunoglobulin release. Nevertheless, other so far unidentified TLR functions in these cell subsets may exist.

In conclusion, we report that TLR expression differs according to the B-cell developmental stage. TLR1–TLR9 may promote augmentation of antibody production by plasma cells, which may have therapeutic application. By contrast, TLR10, for which no ligand has so far been recognized and which is virtually unexpressed in HSC or in plasma cells, may functionally be linked to specific naïve and memory B-cell actions rather than simple pathogen sensing. It is tempting to speculate that TLR10 mediates a unique function (e.g. interaction with self-proteins and thereby homing of naïve and memory B cells to secondary lymphoid organs). This speculation is based on TLR10 being expressed at the cell surface[1] and not by cell lineages other than B cells,[5,6,9,16] B-cell differentiation to plasma cells, requiring (among others) changes in cell-surface proteins and homing,[18] on findings of TLR4 recognizing host structures[21] and of toll guiding dorsal-ventral cell orientation in *Drosophila*.[22]

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Disclosure

There are no financial disclosures for this work.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. Schematic flow-chart of cell isolations and cell purities.

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Latent Membrane Protein 2B Regulates Susceptibility to Induction of Lytic Epstein-Barr Virus Infection▽

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The B-lymphotropic Epstein-Barr virus (EBV) encodes two isoforms of latent membrane protein 2 (LMP2), LMP2A and LMP2B, which are expressed during latency in B cells. The function of LMP2B is largely unknown, whereas LMP2A blocks B-cell receptor (BCR) signaling transduction and induction of lytic EBV infection, thereby promoting B-cell survival. Transfection experiments on LMP2B in EBV-negative B cells and the silencing of LMP2B in EBV-harboring Burkitt's lymphoma-derived Akata cells suggest that LMP2B interferes with the function of LMP2A, but the role of LMP2B in the presence of functional EBV has not been established. Here, LMP2B, LMP2A, or both were overexpressed in EBV-harboring Akata cells to study the function of LMP2B. The overexpression of LMP2B increased the magnitude of EBV switching from its latent to its lytic form upon BCR cross-linking, as indicated by a more-enhanced upregulation and expression of EBV lytic genes and significantly increased production of transforming EBV compared to Akata vector control cells or LMP2A-overexpressing cells. Moreover, LMP2B lowered the degree of BCR cross-linking required to induce lytic EBV infection. Finally, LMP2B colocalized with LMP2A as demonstrated by immunoprecipitation and immunofluorescence and restored calcium mobilization upon BCR cross-linking, a signaling process inhibited by LMP2A. Thus, our findings suggest that LMP2B negatively regulates the function of LMP2A in preventing the switch from latent to lytic EBV replication.

Epstein-Barr virus (EBV) is a ubiquitous B-lymphotropic gammaherpesvirus which persists after primary infection latently in the host for life and may switch periodically to its lytic form (28). In vitro, EBV undergoes very efficient growth transformation and immortalizes infected B cells by latent infection, resulting in lymphoblastoid cell lines (LCLs) expressing a limited number of viral genes, including six viral nuclear antigens (EBNAs) and latent membrane protein 1 (LMP1) and LMP2 (30). The ability to transform B cells implicates EBV as the culprit for a variety of malignancies, including Burkitt's lymphoma, Hodgkin's disease, and posttransplant lymphoproliferative disease (8, 24, 38). In vivo, EBV persists in latently infected memory B cells circulating in the peripheral blood (30). These latently infected cells do not express EBNAs or LMP1, but may express LMP2 (1, 2). Since LMP2 has no transformation capacity (12), this may suggest a pivotal role of LMP2 in the regulation of the balance between latent and lytic EBV.

Transcription of *LMP2* is controlled by two promoters separated in the viral DNA by 3 kb (31). Two mRNAs that have different 5′ exons followed by eight common exons encode two distinct proteins, LMP2A and LMP2B, respectively. LMP2A contains an N-terminal cytoplasmic domain of 119 amino acids with eight tyrosines that are phosphorylated in LCLs, 12 transmembrane domains, and a C-terminal domain of 12 amino acids. LMP2A blocks B-cell receptor (BCR) signal transduction through specific phosphotyrosine motifs in its N-terminal domain and promotes B-cell survival. This function is dependent on the expression level of LMP2A (1, 2, 5, 6, 15, 21, 35). LMP2B lacks the entire N-terminal cytoplasmic domain. A recent work using transfection of LMP2 into EBV-negative cells has suggested possible roles for LMP2B. LMP2B colocalized with LMP2A in the membrane where the C terminus of both splice variants can interact and regulate the activity of each other (17). Furthermore, LMP2B was shown to negatively regulate LMP2A activity by interfering with its aggregation (29). Another study revealed protein domains of LMP2B which are required for intra- and extracellular localization and self-aggregation (37), which raised the question of whether the function of LMP2B in EBV is bound to its localization independently of LMP2A. Nevertheless, whether and how LMP2B is involved in the regulation of latent and lytic EBV infection in B cells harboring the functional virus remains a largely unresolved question.

Burkitt's lymphoma-derived Akata cells provide an optimal model to study the balance between latent and lytic EBV. Specifically, lytic EBV infection can be initiated in Akata cells by cross-linking their BCR using anti-immunoglobulin G (anti-IgG) (36). Importantly, upon induction of lytic EBV infection, the majority of viral genes are expressed (3, 39). Since these EBV genes could have an impact on the function of LMP2B, we used Akata cells to investigate the function of LMP2B in cells harboring functional EBV. Recently, we found that the

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silencing of *LMP2B* reduces susceptibility to induction of lytic EBV infection upon BCR cross-linking (27). This result indicated a role of LMP2B distinct from that of LMP2A in the regulation of EBV lytic activation. In this work, we further pursue the hypothesis that LMP2B exhibits a negative-regulatory effect on LMP2A maintenance of EBV latency. Thus, we compared the effects of overexpression of LMP2B and LMP2A on the susceptibility to induction of lytic EBV infection and on cellular signaling pathways in Akata cells.

MATERIALS AND METHODS

Cell lines and primary cells. The Burkitt's lymphoma cell line Akata (36) was grown in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Akata cells were a kind gift of A. Bell (Birmingham, United Kingdom). Akata cells transfected with the plasmids pEneo (33), pEneo-LMP2A (27), and pEneo-FLAG-LMP2B, named Akata-vector (27), Akata-LMP2A (27), and Akata-LMP2B pools 1 to 3, respectively, were cultured in the same medium supplemented with 0.4 mg/ml G418 (Promega, Mannheim, Germany). Akata-cre cells that stably overexpress the regulatable creER^{T2} recombinase (9) integrated into the vector pcDNA3.1 were cultured in the same medium supplemented with 0.4 mg/ml G418 (Promega, Mannheim, Germany). Cord blood mononuclear cells (CBMC) were obtained from heparinized blood by Ficoll-Hypaque gradient centrifugation (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland) and washed with phosphate-buffered saline (Gibco, Invitrogen Life Sciences, Basel, Switzerland). Informed consent was obtained from parturient women. The Zurich institutional ethics committee approved the collection and use of clinical material. B95.8 cells were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (23).

Plasmids. The sequence coding for LMP2B was PCR amplified from the vector pSG5-LMP2 (15, 31) with following primers, including cloning adapters and 3× FLAG for LMP2B tagging: FLAG-LMP2B-F (5′-CGCGTTTAAACAT GGACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGAT TACAAGGATGACGATGACAAGAATCCAGTATGCCTGCCTG-3′) and LMP2-Rev (5′-GCGCTCGAGTTATACAGTGTTGCGATATGGGGTC-3′). The PCR product was cloned via PmeI/XhoI into the Moloney murine leukemia virus-derived pEneo bicistronic expression vector containing an internal ribosome entry site with a neomycin selection marker (33), resulting in the vectors pEneo-FLAG-LMP2B and pEneo-LMP2A (27). Positive clones were verified by sequencing. The parental pEneo plasmid was used as the control vector.

Transfection. Cells (1×10^6) were electroporated with 2 μg of the pEneo, pEneo-LMP2A, or pEneo-FLAG-LMP2B plasmid with Nucleofector II (Amaxa GmbH, Cologne, Germany) with Buffer T and the program A-23. Electroporated cells were allowed to recover for 2 days and were then selected with 0.8 mg/ml G418 (Promega) for 2 to 3 weeks until resistant cells arose. These cells were named Akata-vector, Akata-LMP2A, and Akata-LMP2B pools 1 to 3, respectively. One month after selection, cells were supplemented with 0.4 mg/ml G418 and used for experiments. To generate double-transfected Akata cells, either stable Akata-LMP2B cells were electroporated transiently ($_t$) with the vector pEneo-LMP2A (named 2B + A_t) or Akata-LMP2A cells were electroporated transiently with the vector pEneo-FLAG-LMP2B (named 2A + B_t).

Immunoblot analysis. Total cellular protein extracts were prepared by disruption of cells in radioimmunoprecipitation assay lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 50 mM Tris, pH 7.5). After denaturation for 10 min in 4× loading LDS buffer (Invitrogen, Basel, Switzerland), samples were separated on a 10% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane (Schweitzer & Schell Bioscience GmbH, Dassel, Germany). Membranes were blocked with 5% low-fat milk in 1× phosphate-buffered saline (PBS)–0.1% Tween 20 and incubated with either mouse anti-FLAG M2 (Sigma, St. Louis, MO), rat anti-LMP2A (clone 14B7) (5), or anti-c-*myc* (A-14; Santa Cruz Biotech, Inc., Santa Cruz, CA) antibody overnight at 4°C. Immunoreactive proteins were detected by the secondary antibodies against mouse and rabbit, respectively (Cell Signaling Technology, Danvers, MA) and an enhanced chemiluminescence detection kit (SuperSignal West Femto; Perbio Science Switzerland S.A., Lausanne, Switzerland).

IP. Total cellular protein extracts were prepared by disruption of 5×10^6 cells in Tris nondenaturing lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 50 mM Tris, pH 7.5), 2 mM NaF, and 2 mM Orthovanadate (all from Sigma). Isolated proteins were immunoprecipitated with a Sigma immunoprecipitation (IP) kit according to the manufacturer's instructions. IPs were performed with the same antibodies used for immunoblotting or without an antibody.

Immunostaining. Cells (5×10^4) were centrifuged by Cytospin on double Cytofunnel glass slides (Thermo, Waltham, MA) and fixed with methanol for 10 min at −20°C. Cells were permeabilized with 1% Triton X-100 in 1× PBS for 20 min at room temperature. Immunoreactive proteins were detected with a rat anti-LMP2A antibody, 14B7 (1:100), and a secondary Alexa 488-labeled goat anti-rat IgG antibody diluted 1:200 (Molecular Probes, Invitrogen). FLAG-tagged LMP2B was detected with anti-FLAG M2 from Sigma (1:500) and a secondary Alexa 594-labeled goat anti-mouse IgG antibody diluted 1:200 (Molecular Probes, Invitrogen). BZLF1 was detected with anti-BZLF1 (Argene, North Massapequa, NY) diluted 1:100 and probed with the same secondary antibody used for FLAG detection.

Flow cytometry. To evaluate the surface IgG (sIgG) content of transfected cells for later stimulation experiments with BCR cross-linking, cells were fixed in 4% paraformaldehyde (Sigma) and stained with a phycoerythrin-labeled anti-human IgG1,κ antibody (BD Biosciences, Basel, Switzerland). A phycoerythrin-labeled anti-mouse IgG1,κ antibody (BD Biosciences) was used as an isotype control. To determine the percentage of cells in which EBV was activated by BCR cross-linking, 0.5×10^6 cross-linked and non-cross-linked cells were stained with a fluorescein isothiocyanate (FITC)-labeled antiviral gp350/220 antibody diluted 1:10 (Biodesign International, Saco, ME) and analyzed by flow cytometry (FC-500; Beckmann-Coulter, Krefeld, Germany).

BCR cross-linking of Akata cells. Akata cells were split to 0.5×10^6 cells/ml 24 h before BCR cross-linking. Cells (0.5×10^6/ml) were then BCR cross-linked with 0.1 μg/μl polyclonal rabbit anti-human-IgG (Dako, Zug, Switzerland) for 3 h and suspended afterwards in fresh RPMI 1640. Cross-linked and non-cross-linked cells were collected for subsequent analyses.

qRT-PCR. Total RNA was extracted from 0.5×10^6 cells with an RNeasy kit from Qiagen (Hombrechtikon, Switzerland). DNase [DNA-free; Ambion (Europe), Huntington, Cambridgeshire, United Kingdom] treatment was performed before cDNA synthesis with an Omniscript RT kit (Qiagen). Quantitative real-time PCR (qRT-PCR) was done with validated TaqMan systems for the housekeeping gene *HMBS* and the lytic EBV genes *BZLF1*, *BXLF1*, and *LMP2* (14) on an ABI 7200 (Applied Biosystems). TaqMan data were analyzed using SDS 2.2 (Applied Biosystems), and mRNA expression was normalized to *HMBS* mRNA, resulting in threshold cycle (ΔC_T) values. ΔC_T values were further normalized by dividing "cross-linked" by "not cross-linked" values, resulting in $\Delta\Delta C_T$ values. qRT-PCR data for control vector $\Delta\Delta C_T$ were set to 100% and LMP2B- or LMP2A-overexpressing cells were compared to it.

Transformation assay. Fifty thousand freshly isolated CBMC per well were seeded in a 96-well plate. Culture supernatants were filtered through a 0.45-μm polyvinylidene difluoride Millex-HV filter (Millipore Corporation, MA). Fifty microliters of filtered culture supernatant of cells 24 h after anti-IgG cross-linking or no cross-linking was added to a final volume of 100 μl per well. A total of 10 wells for each filtered culture supernatant was plated and inoculated. Filtered supernatant from B95.8 served as the positive control, whereas the negative control was CBMC cultured with medium only (23). The transformation capacity was monitored by counting the wells after 6 weeks, when growth and clustering of cells could be observed (34). The percentage of transformation was calculated by setting 10 transformed wells to 100% and by normalization to the transformation capacity of the supernatant of cells that were not cross-linked, representing the spontaneous activation of EBV in Akata cells. Statistics were done with Prism 4 (GraphPad Software, Inc.).

Calcium mobilization. Cells (5×10^6) were stained with Fluo-3 (6 μM final concentration) and Fura-Red (15 μM final concentration; both from Invitrogen) for 45 min in RPMI 1640 at 37°C. After a washing step with 1× PBS, cells were stored at room temperature in the dark. Before measurement, cells were incubated for 5 min at 37°C. Calcium mobilization was measured by adding ionomycin (2 μg/ml final concentration; Invitrogen) as the control and anti-IgG at the same concentration as in stimulation experiments (100 μg/ml) by using a FC-500 (Beckmann-Coulter) with an argon laser at 488 nm. Fluorescent emission was recorded at 520 nm (Fluo-3) and 670 nm (Fura-Red), and the Fluo-3/Fura-Red ratio was plotted against time. The baseline was recorded prior to anti-IgG addition for 30 s and for 5 min after BCR cross-linking. The increase over the baseline level was calculated for the time of peak of calcium mobilization (t_p) by using FlowJo 5.7.2 software. The percentage of responding cells was calculated for the time slice from t_p to $t_{p\ +\ 2\ min}$. As an additional control, Akata-cre cells were stimulated and the calcium mobilization was measured.

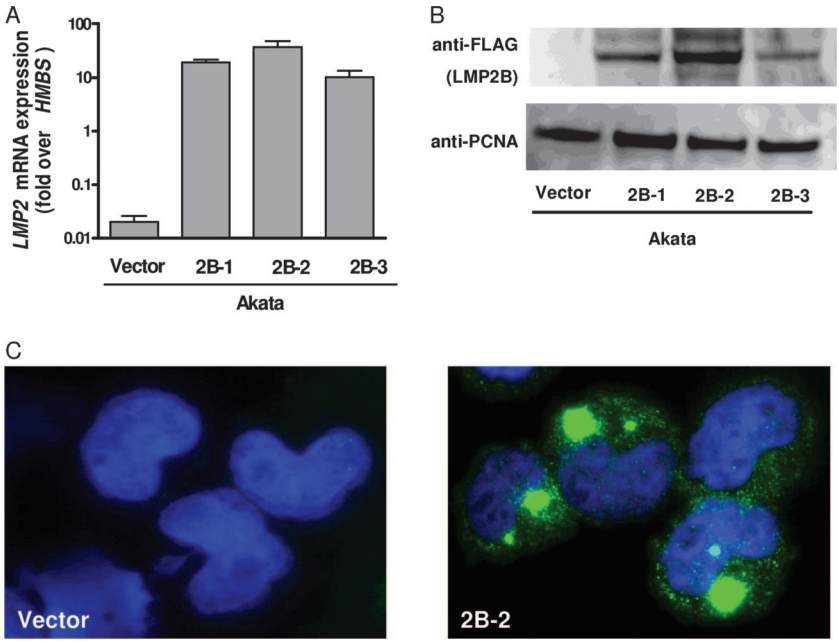

FIG. 1. Generation of LMP2B-overexpressing Akata cells. (A) Overexpression of LMP2B pools 1 to 3 (2B-1, 2B-2, 2B-3) by qRT-PCR using specific TaqMan systems targeting *HMBS* and *LMP2* mRNA, respectively. (B) Immunoblot of FLAG-LMP2B pools 1 to 3 (2B-1, 2B-2, 2B-3). (C) Immunostaining of FLAG-LMP2B in Akata-vector control cells and Akata-LMP2B pool 2 (2B-2).

RESULTS

Construction of LMP2B-overexpressing Akata cells. In order to investigate the effects of LMP2B on LMP2A and the switch from latent to lytic EBV replication, we first constructed EBV-harboring Akata cells overexpressing either LMP2B or LMP2A (27) and Akata cells with the vector control (27). Given that no specific antibody against LMP2B exists, we chose to tag LMP2B with a 3× FLAG sequence at the N terminus. The tag was placed at the N terminus, since it has been suggested that clustering of LMP2A and LMP2B occurs over the common C termini and that LMP2B influences the activity of its LMP2A isoform only when they colocalize (18, 29). As the transfection efficiency of B cells is low with common protocols, we decided to establish Akata cell pools stably overexpressing LMP2B, LMP2A, or a control vector. Thus, Akata cells were transfected independently with pEneo-FLAG-LMP2B, pEneo-LMP2A, or the control vector pEneo alone, as described in Materials and Methods. After neomycin selection, stable overexpression of LMP2B was verified for all three independently transfected Akata cell pools at the RNA level by qRT-PCR and at the protein level by immunoblotting and immunostaining. The three LMP2B-overexpressing cell pools (Akata-LMP2B pools 1, 2, and 3) showed different levels of overexpression of LMP2B after transfection (Fig. 1A). The mRNA expression levels correlated with protein levels, whereby Akata-LMP2B pool 1 showed a medium level, pool 2 a high level, and pool 3 the lowest level of LMP2B mRNA and protein, respectively (Fig. 1B). Immunostaining was done with Akata-LMP2B pool 2. Most overexpressed LMP2B localized to cytosolic compartments, whereas smaller amounts were detected in the plasma membrane (Fig. 1C). Furthermore, all cell lines were stained for sIgG and were found to express sIgG in similar percentages (Table 1). Thus, similar prerequisites for susceptibility to stimulation by BCR cross-linking with anti-IgG were ensured in the distinct cell lines.

LMP2B overexpression increases the magnitude of EBV lytic activation after BCR cross-linking. To assess the distinct effects of LMP2B and LMP2A on lytic activation of EBV in

TABLE 1. Characteristics of Akata cells used[a]

Cell line	Vector construct	% sIgG$^+$ cells	% Cells activated by gp350/220 staining		
			0 h	24 h	
				Cross-linked	Not cross-linked
Akata[b]	None	96 ± 1	ND	ND	ND
Akata-vector[b]	pEneo	92 ± 2	0.30 ± 0.11	4.62 ± 0.06	0.58 ± 0.09
Akata-LMP2B[c]	pEneo-FLAG-LMP2B	88 ± 4	0.36 ± 0.20	5.42 ± 0.94	0.69 ± 0.23
Akata-LMP2A[b]	pEneo-LMP2A	92 ± 1	0.24 ± 0.05	1.00 ± 0.81	0.34 ± 0.13

[a] ND, not detected.
[b] Values are presented as means ± SD from three independent experiments.
[c] Values are presented as means ± SD from pools 1 to 3.

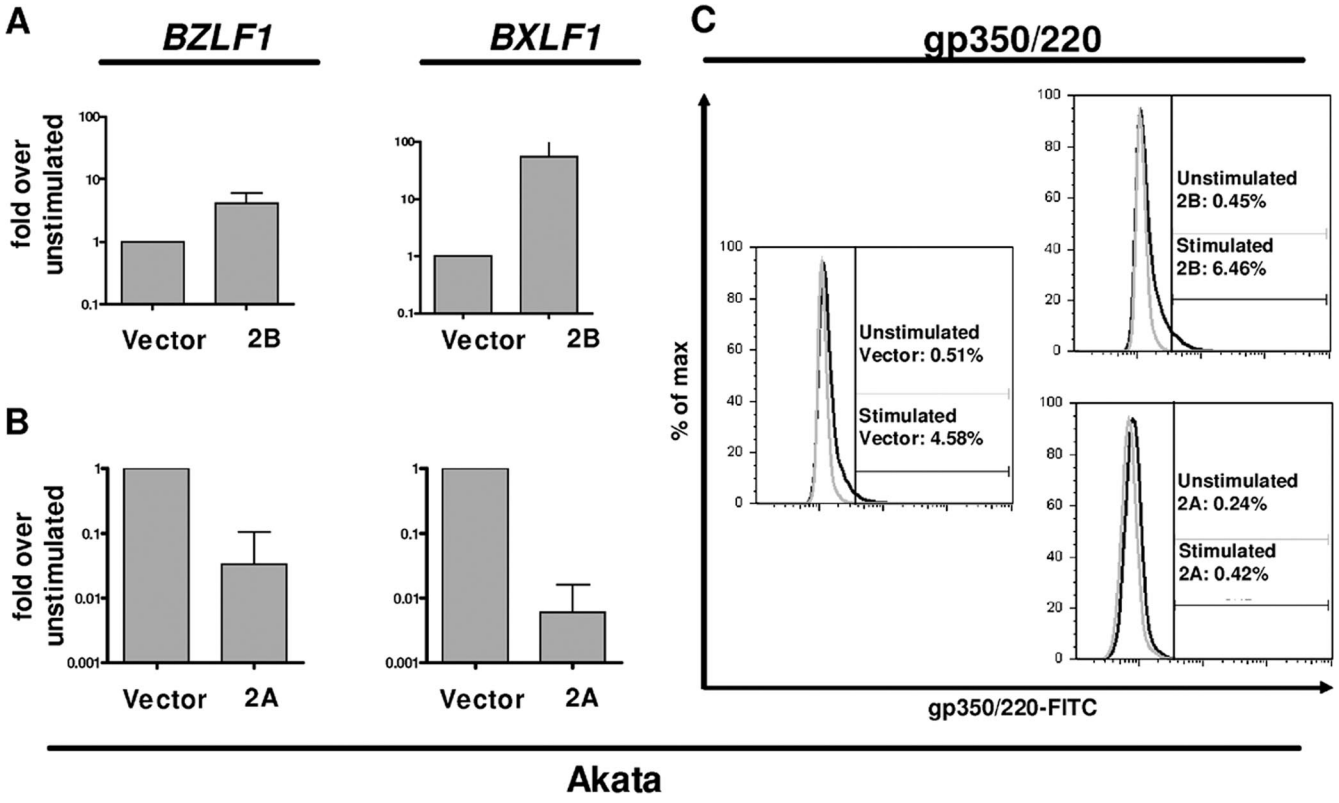

FIG. 2. LMP2B overexpression increases the magnitude of EBV lytic activation after BCR cross-linking, whereas LMP2A overexpression results decreased magnitude. qRT-PCR with specific systems for *HMBS*, *BZLF1*, and *BXLF1* mRNA, respectively, for Akata-vector control, Akata-LMP2B (A), and Akata-LMP2A (B) 24 h after BCR cross-linking. Means and standard deviations (SD) of qRT-PCR results are from three independent stimulation experiments on one representative polyclonal population. (C) Flow cytometry for gp350/220-FITC-labeled unstimulated (gray line) or stimulated (black line) cells 24 h after BCR cross-linking. One representative measurement is shown for Akata-vector control, Akata-LMP2B, and Akata-LMP2A cells with gp350/220-FITC-positive cells gated and indicated as percentages. Means and SD for three independent experiments are summarized in Table 1.

Akata cells, we stimulated Akata-LMP2B cells or Akata-LMP2A cells and the corresponding Akata-vector control cells by BCR cross-linking. After 24 h, cross-linked and non-cross-linked cells were collected and examined by qRT-PCR for expression of the immediate-early lytic gene *BZLF1* and the early lytic gene *BXLF1* encoding the viral thymidine kinase. The data were normalized to *HMBS* mRNA expression and are presented as ratios of cross-linked to non-cross-linked cells (Fig. 2). Akata-LMP2B cells showed mRNA expression levels of *BZLF1* and *BXLF1* that increased 4-fold and 55-fold, respectively (Fig. 2A). By contrast and as expected, transcription levels of lytic EBV genes were reduced in Akata-LMP2A cells, where expression of *BZLF1* and *BXLF1* mRNAs was reduced by 97% and 99%, respectively (Fig. 2B). To confirm these results at the protein level, we stained cells before and 24 h after BCR cross-linking with a FITC-labeled antiviral gp350/220 antibody in three independent stimulations. Indeed, as determined by flow cytometry, Akata-LMP2B cell pools expressed up to 5.4-fold- and 4.6-fold-higher gp350/220 levels than Akata-LMP2A cells and Akata-vector control cells, respectively (Fig. 2C; Table 1).

LMP2B-overexpressing Akata cells produce more infectious EBV than control cells upon BCR cross-linking. To verify the complete activation of the EBV lytic cycle following BCR cross-linking, the production of infectious EBV was monitored by the transformation of primary human B cells (34). Following BCR cross-linking of Akata-LMP2B, Akata-LMP2A, and Akata-vector control cells, supernatants were prepared from three independent experiments and added to freshly isolated CBMC to determine the transformation capacity of the infectious EBV produced. After normalization to non-cross-linked cells, the transformation capacities of supernatants from Akata-vector control cells were 67%, but those from Akata-LMP2B-cell pools were up to 100% ($P = 0.0356$), in contrast to the 0% transformation capacity of supernatants from Akata-LMP2A cells ($P = 0.0089$; Fig. 3).

Overexpression of LMP2B decreases the degree of BCR stimulation required to induce lytic EBV infection, in contrast to overexpression of LMP2A. LMP2A blocks BCR signaling, thereby impeding EBV lytic activation (6). It is not clear how the expression level of LMP2A influences the degree of BCR cross-linking (BCR activation) required to induce lytic EBV infection and how this is affected by LMP2B overexpression. Therefore, we assessed the magnitude of EBV lytic activation as a function of the anti-IgG dose to engage BCR by cross-linking Akata-vector, Akata-LMP2B, or Akata-LMP2A cells with increasing concentrations of anti-IgG. To quantify the activation of EBV lytic infection, mRNA expression of *BZLF1*

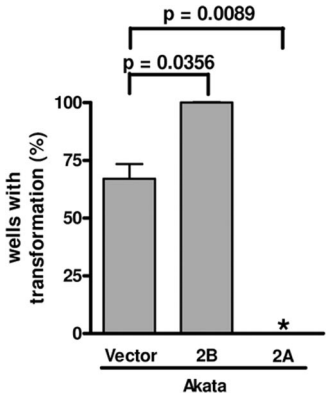

FIG. 3. More infectious EBV is produced in LMP2B-overexpressing Akata cells than in the control. Isolated CBMC were infected with supernatant from Akata-vector control, Akata-LMP2A, or Akata-LMP2B pools 1 to 3 collected 24 h after stimulation by BCR cross-linking. The transformation capacity was monitored by counting the wells after 6 weeks, at which time growth and clustering of cells indicated transformation. The percentage of transformation was calculated by setting 10 wells showing signs of transformation to 100% and by normalization to the transformation capacity of the supernatant of the corresponding BCR non-cross-linked control cells, representing the spontaneous EBV lytic activation in Akata cells. Means and SD are from three independent stimulation experiments with subsequent collection of supernatant and infection of CBMC. *t* tests were performed with a 95% confidence interval. *, no wells showed signs of transformation.

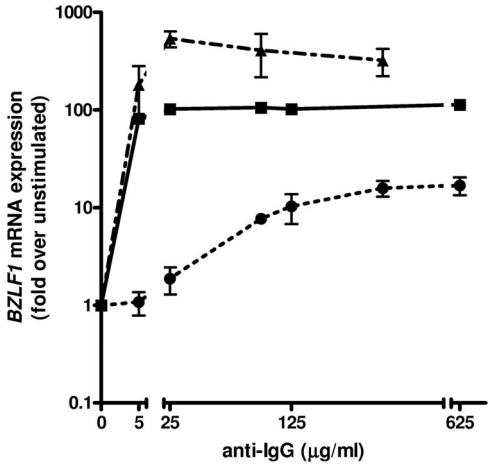

FIG. 4. Overexpression of LMP2B decreases the degree of BCR stimulation required to induce lytic EBV infection, in contrast to overexpression of LMP2A. *BZLF1* mRNA expression in Akata-vector control (■), Akata-LMP2A (●), or Akata-LMP2B (▲) 24 h after BCR cross-linking using increasing doses of anti-IgG. Means and SD are from three independent stimulation experiments on one representative polyclonal population.

at 24 h after BCR cross-linking was measured (Fig. 4). Cross-linked Akata-vector control cells compared to non-cross-linked control cells showed an almost 100-fold increase of *BZLF1* mRNA expression with the lowest anti-IgG concentration of 5 μg/ml and around 100-fold-higher peak *BZLF1* mRNA expression levels with anti-IgG concentrations of 25 μg/ml or higher. Akata-LMP2B cells showed a similar but greater increase in *BZLF1* mRNA expression and around five-fold-higher peak levels than Akata-vector cells with anti-IgG concentrations of 25 μg/ml or higher. By contrast, Akata-LMP2A cells required anti-IgG concentrations of at least 25 μg/ml to show an increase in *BZLF1* mRNA expression and an anti-IgG concentration of 625 μg/ml to show maximal *BZLF1* mRNA expression levels, which were around 10-fold lower than peak expression levels in Akata-vector cells. Thus, cells with higher expression levels of LMP2A required higher doses of anti-IgG to induce an EBV lytic activation, which was still of a considerably lower magnitude than that for Akata-vector control cells. These results suggest that the expression level of LMP2A has an impact on the amount of BCR cross-linking required to induce lytic EBV infection and that higher expression levels of LMP2A can be overridden, though only partially, with a higher degree of BCR cross-linking. On the other hand, higher LMP2B expression levels seemed to lower the degree of BCR cross-linking required to induce EBV lytic activation and to increase the magnitude of inducible EBV lytic activation.

LMP2B physically interacts with LMP2A before and after BCR cross-linking in Akata cells. To investigate whether overexpressed LMP2B physically interacts with endogenous LMP2A, we isolated whole-cell protein extracts from Akata-LMP2B pool 2 (2B-2) and Akata-vector control cells with a subsequent pull-down IP of LMP2A. Subsequently, we performed immunoblotting against FLAG tags and LMP2A (Fig. 5A). Indeed, coimmunoprecipitated LMP2B was detected by anti-FLAG in Akata-LMP2B cells but not in Akata-vector control cells, whereas endogenous LMP2A was detected in both. Control IPs, with an anti-c-*myc* antibody or without antibody and subsequent immunoblotting against c-*myc* or FLAG (Fig. 5B and C) showed no unspecific pull-down, confirming the specificity of the IPs, despite the overexpression of FLAG-LMP2B.

Next, to elucidate in which compartment LMP2B and LMP2A localize, we transfected Akata-LMP2A (2A) transiently with FLAG-LMP2B (B_t) (indicated as 2A + B_t) and immunostained LMP2A or FLAG-LMP2B for fluorescent microscopy. The images shown in Fig. 6B suggest partial colocalization of both LMP2 isoforms in the same cellular compartments (Fig. 6B). Additionally, we found an accumulation of LMP2B in the cytosolic region, as seen in stable Akata-LMP2B and described above (Fig. 1C). To determine if there is a relocalization of LMP2A or LMP2B upon BCR cross-linking, we double-stained Akata-LMP2A cells transiently transfected with FLAG-LMP2B (2A + B_t) for FLAG-LMP2B and for LMP2A (Fig. 6C), and for BZLF1 and LMP2A (Fig. 6D) after BCR cross-linking. An immunofluorescence analysis indicated a rather modest shift of LMP2A and LMP2B into the cytosol after BCR cross-linking, but still-adequate amounts of both LMP2s were located in the same compartments as before BCR cross-linking.

LMP2B restores calcium mobilization in LMP2A-overexpressing Akata cells. It has been previously established that LMP2A blocks calcium mobilization induced by BCR cross-linking (20–22). To investigate the impact of LMP2B overexpression on calcium mobilization, we determined the calcium levels before (baseline) and after BCR cross-linking, monitoring the kinetics for 5 min in Akata-vector control, Akata-LMP2B pool 2 (2B-2), and Akata-LMP2A (2A) cells, respectively. Additionally, Akata-cre cells overexpressing creER^{T2}

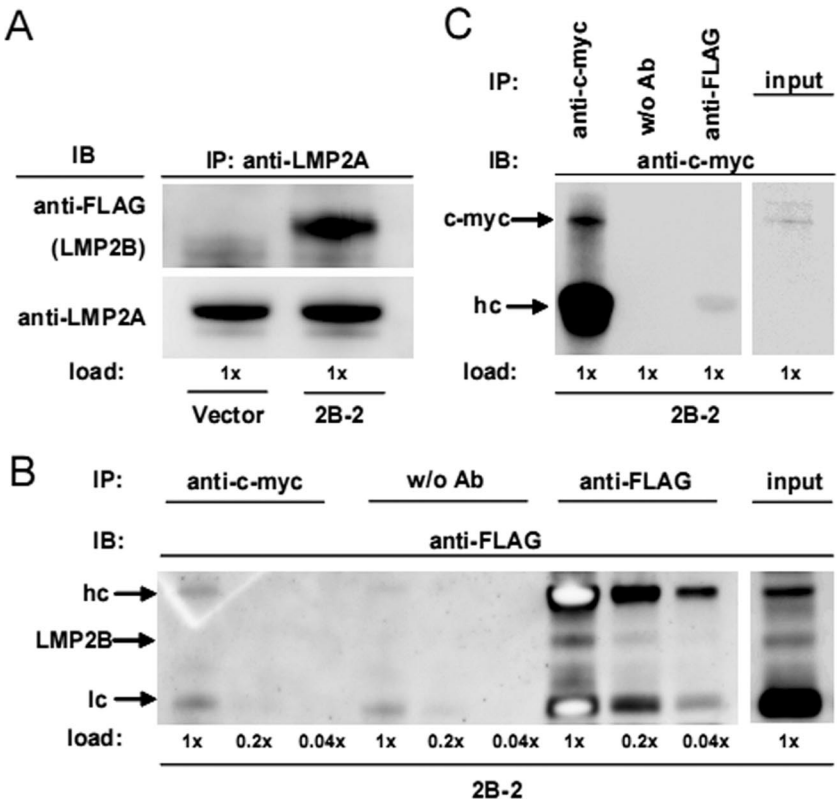

FIG. 5. LMP2B coimmunoprecipitates with LMP2A. Whole-cell protein lysates of Akata-LMP2B pool 2 (2B-2) and the Akata-vector control were immunoprecipitated (IP) with anti-LMP2A (A). The IPs were separated on a SDS gel and immunoblotted (IB) against FLAG and LMP2A. The heavy band in the LMP2A immunoblot represents the heavy Ig chain of the mouse antibody used for pull-down, whereas the lower, narrow band represents the equal amount of LMP2A pulled down in 2B-2 and vector input lysates. (B) The input lysate of 2B-2 was split into IPs without antibody, against c-*myc*, and FLAG and immunoblotted (three dilutions; 1×, 0.2×, 0.04×) against FLAG. No unspecific pull-down with anti-c-*myc* was observed due to the overexpression of FLAG-LMP2B. (C) The same IPs as those used in panel B were loaded on a SDS gel and immunoblotted against c-*myc* to verify that the IP was working. The upper band (65 kDa) and the lower band (63 kDa) in the input lysate of 2B-2 represent two forms of c-*myc*.

recombinase in the cytosol were stimulated and monitored in parallel to exclude any epiphenomena due merely to overexpression which could influence calcium mobilization. Calcium mobilization reached up to 3-fold, 3.3-fold, and 3.5-fold peaks compared to baseline levels after BCR cross-linking in Akata-vector control cells, Akata-cre cells, and Akata-LMP2B cells, respectively (Fig. 7). Next, we generated double transfectants by electroporation of Akata-LMP2B cells transiently with the vector pEneo-LMP2A (2B + A_t) as described in Materials and Methods and measured the calcium mobilization after BCR cross-linking. We observed not only a decrease of calcium mobilization from 3.5-fold to 1.9-fold in Akata-LMP2B cells but also a decrease of responding cells from 90% to 74%. These results are in agreement with previous studies (20–22). The calcium mobilization in Akata-LMP2A cells revealed the same low calcium mobilization and percentage of responding cells as observed in the double-transfected 2B + A_t cells (1.8-fold and 77%, respectively). To address the question of whether it is possible to rescue the phenotype of Akata-vector control cells, we transiently transfected Akata-LMP2A cells with the vector pEneo-FLAG-LMP2B (2A + B_t). Interestingly, the calcium mobilization was restored to Akata-vector control cell levels from 1.8- to 2.9-fold after BCR cross-linking. Additionally, responding cells increased from 77% to 82% in double-transfected 2A + B_t cells (Fig. 7).

DISCUSSION

In this work, we investigated the impact of LMP2B on the potential of LMP2A to maintain EBV in its latent state. We demonstrate that LMP2B increases the magnitude of EBV switching from its latent to its lytic form upon BCR cross-linking, lowers the degree of BCR cross-linking required to provoke this switching, and is involved in augmenting signaling via calcium mobilization upon BCR cross-linking in Akata cells harboring functional EBV. These observations suggest a negative regulatory effect of LMP2B on the ability of LMP2A to block BCR signaling, thereby preventing EBV from switching from latent to lytic infection in B cells.

Although LMP2A and LMP2B are similar in their structure, the lack of the signaling amino-terminal domain in LMP2B indicates distinct functions for these two proteins. Indeed, the overexpression of LMP2B in Akata cells resulted in higher mRNA expression of immediate-early and early lytic EBV genes upon BCR cross-linking, whereas the overexpression of LMP2A results in lower mRNA expression in these genes.

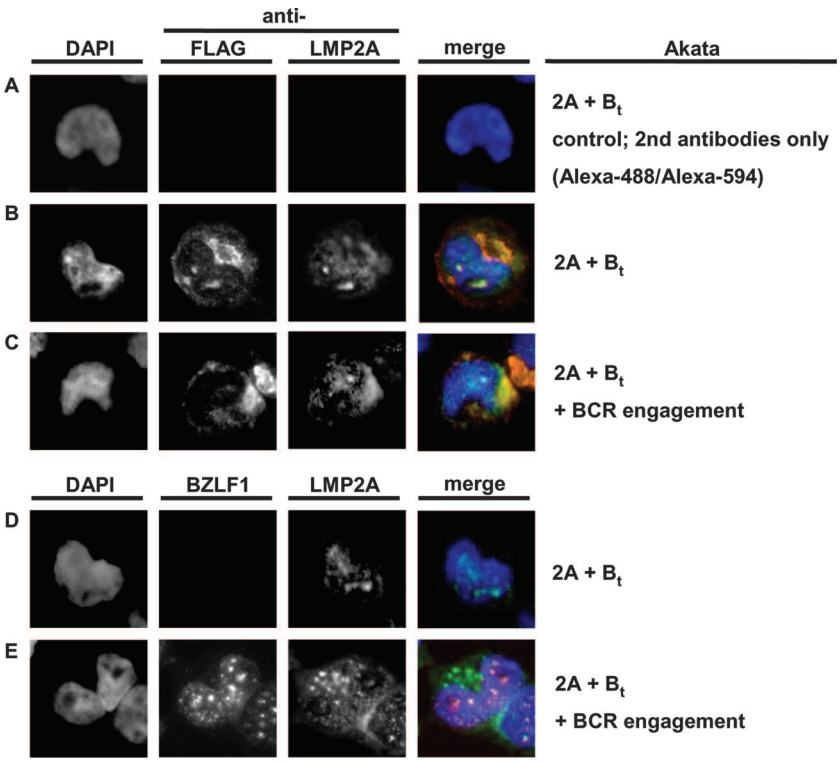

FIG. 6. Overexpressed LMP2B and LMP2A colocalize before and after BCR cross-linking. (A) Negative control for secondary antibodies. (B) To investigate where LMP2B and LMP2A localize, we transiently transfected Akata-LMP2A cells with FLAG-LMP2B (2A + B_t) and stained for LMP2A and FLAG-LMP2B. To determine if there occurs a relocalization of LMP2A or LMP2B upon BCR cross-linking, we stained the double-transfected Akata cells (2A + B_t) for FLAG-LMP2B and for LMP2A (C) and for BZLF1 and LMP2A (D) 3 h after BCR cross-linking (E). DAPI, 4′,6′-diamidino-2-phenylindole.

Moreover, LMP2B overexpression leads as well to production of more EBV envelope protein gp350/220 and functional virus than do LMP2A-overexpressing or control Akata cells upon BCR cross-linking. These findings are compatible with our previous observation that silencing of *LMP2B* in Akata cells reduces the susceptibility of these cells to undergo EBV lytic activation induced by BCR cross-linking (27). Thus, our current and previous data provide evidence that LMP2B is involved in the regulation of EBV switching from latent to lytic infection EBV in B cells harboring the whole virus in its latent form.

The overexpression of LMP2B did not result in spontaneous switching of latent to lytic EBV. Nevertheless, the higher magnitude of EBV lytic activation in LMP2B-overexpressing Akata cells than that in control Akata cells upon BCR cross-linking with similar doses of anti-IgG suggested that LMP2B exerts its mode of action through lowering the required degree of BCR cross-linking and thus BCR signaling needed. Since LMP2A blocks BCR signaling (5, 6, 12, 15, 21, 29, 30, 35), we addressed the fundamental question of whether the magnitude of EBV lytic activation at given expression levels of the LMP2s depends on the dose of anti-IgG required to cross-link BCR, i.e., the degree of BCR cross-linking. Indeed, although increasing doses of anti-IgG elevated the levels of activation of lytic EBV in LMP2A-overexpressing Akata cells until they reached a plateau, peak levels of induced lytic EBV in these cells were around at least 10-fold lower than in control Akata cells or around 30- to 50-fold lower than in LMP2B-overexpressing Akata cells. This result suggests that both endogenous and overexpressed LMP2A is able to reduce BCR signaling very effectively and cannot be completely counteracted, even by saturated levels of anti-IgG added for cross-linking. Thus, the amount of overexpressed LMP2B was able to decrease the activity of endogenous LMP2A on BCR signaling but could not abolish it completely in whole-EBV-containing cells.

An important question to be addressed was if there is an interaction of LMP2A and LMP2B in Akata cells harboring EBV and, if so, where the two isoforms of LMP2 colocalize. The physical interaction between LMP2A and LMP2B was verified by pulling down FLAG-LMP2B with endogenous LMP2A. Our immunostaining results suggest an accumulation of LMP2B in intracellular compartments and to a lesser extent on the plasma membranes of Akata cells harboring whole EBV. Lynch et al. (17) reported that transiently expressed LMP2B localized to perinuclear regions and colocalized with transiently or constitutively expressed LMP2A in EBV-negative BJAB cells or LCL B95-8CR, respectively. Studies using HEK 293 cells overexpressing full-length or deletion mutants of LMP2A revealed a clustering signal of LMP2A at the C terminus leading to homodimerization (18). As LMP2A and LMP2B share eight exons and the C terminus, Rovedo and Longnecker (29) hypothesized that LMP2B colocalizes with LMP2A, forming heterodimers, and subsequently negatively regulates LMP2A activity, leading to decreased degradation of

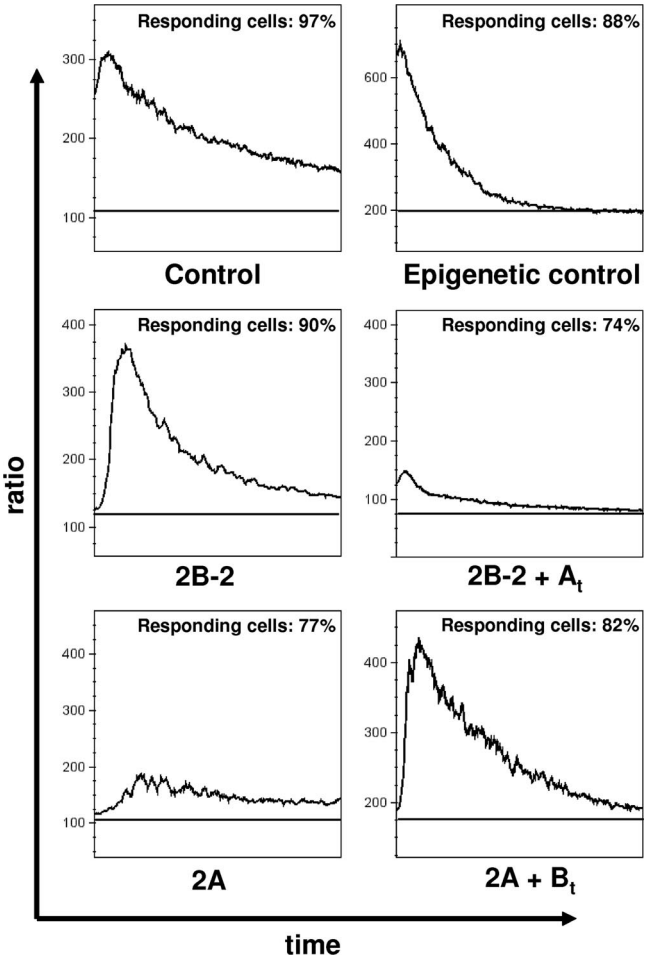

FIG. 7. LMP2B restores calcium mobilization in LMP2A-overexpressing Akata cells. Calcium levels were determined before and after BCR cross-linking, and the kinetics were measured for 5 min. Upper panels, calcium mobilization after BCR cross-linking in Akata control cells and, as an additional control, in Akata cells with epigenetic overexpression of an unspecific protein in the cytosol (see Materials and Methods). Middle panels, calcium mobilization after BCR cross-linking in Akata-LMP2B cells without (2B) and with (2B + A_t) transiently transfected LMP2A. Lower panels, calcium mobilization after BCR cross-linking in Akata-LMP2A cells without (2A) and with (2A + B_t) transiently transfected FLAG-LMP2B. Baselines were measured just before BCR cross-linking. The percentage of responding cells was calculated as described in Materials and Methods.

the tyrosine kinase Lyn. A more-recent study showed that when tagged LMP2B was overexpressed in BJAB cells or HEK 293T cells, LMP2B was found exclusively in intracellular perinuclear compartments (29, 37), a result which is in apparent contrast with the results of the aforementioned study. When LMP2B was truncated at any domain, it resulted in localization to the cell surface. Based on these results taken together, one can hypothesize that the major impact of LMP2B on LMP2A takes place in endosomes, where it interferes either with the activity of LMP2A and subsequent ubiquitination and degradation of Lyn or with the trafficking of LMP2A back to the plasma membrane.

An immunofluorescence analysis of LMP2A and LMP2B suggested a colocalization of LMP2A and LMP2B in Akata cells overexpressing both proteins. We detected a rather modest shift of LMP2B and LMP2A into the cytosol, which suggests their internalization after BCR cross-linking. Nevertheless, a large amount of both LMP2s remains detectable on the plasma membrane, demonstrating an intact turnover process. Moreover, this experiment allowed us to exclude the possibility of misfolded and degraded protein in the endoplasmic reticulum due to overexpression, as has been reported in earlier studies (10, 19). It is known that LMP2A aggregates in lipid rafts, assembling as a signalosome which enables a transient interaction with the tyrosine kinases Syk and Lyn with a subsequent block of the BCR signal (5–7, 15, 16, 21, 25, 26, 35). One can hypothesize that after BCR cross-linking, the anti-IgG/BCR complex is internalized together with closely located lipid rafts and LMP2A signalosomes. If there is an additional function of LMP2A in preventing the whole complex from being transported again to the cell membrane, in this way blocking continuous stimulation, one can hypothesize that there is a loss of LMP2A at the cell surface and an accumulation in endosomes located in the cytosol after BCR cross-linking. LMP2B, which is found in cytosolic compartments, may intervene in this step, disrupting homodimerized LMP2A and restoring the turnover.

As demonstrated here for the first time, calcium mobilization upon BCR cross-linking is dependent on the expression level of LMP2B in EBV-harboring Akata cells. As expected from previous reports (20–22), we observed virtually no calcium mobilization in LMP2A-overexpressing cells upon BCR cross-linking. By contrast, after transient transfection of LMP2B into LMP2A-overexpressing Akata cells, calcium mobilization after BCR cross-linking is increased to levels comparable to those observed for Akata cells overexpressing LMP2B. Conversely, we measured a reduced calcium mobilization in LMP2B-overexpressing Akata cells transiently transfected with LMP2A. As transiently transfected vectors expressing the gene of interest lead to high levels of protein, the dominant effect on the stably transfected Akata cells was predictable. Three different signaling pathways which are activated upon BCR cross-linking in Akata cells have been analyzed: (i) calcium mobilization through phosphatidylinositol 3-kinase, (ii) c-Jun N-terminal kinase activation through Syk and Lyn signaling, and (iii) ERK1/2 phosphorylation through the RAS protein (4). Nevertheless, whether LMP2B is involved partially or throughout all these signaling cascades, taking a key regulatory function upstream, remains unknown. Rovedo and Longnecker showed recently that in the EBV-negative B-cell line BJAB, ectopically expressed LMP2B decreases the activity of LMP2A by alteration of the phosphorylation status (29). Thus, LMP2B would function at an initial step of BCR signaling to restore BCR signal transduction which was blocked by LMP2A. As a more downstream read-out, they chose to measure the calcium mobilization in BJAB cells upon BCR cross-linking. As has previously been shown, the overexpression of LMP2A nearly abolished calcium mobilization (20–22). According to the hypothesis of BCR signal restoration by LMP2B, ectopic overexpression of both splice variants of LMP2 together resulted in the phenotype of BJAB transfected with vector control only.

LMP2B may have important functions not only in the modulation of latent and lytic EBV infection in tumor cells or

memory B cells in the periphery. As was reported previously, LMP2A is expressed in newly infected naïve B cells before latency is established and serves as a tonic signal for survival. This scenario might be true for B cells with crippled or a total loss of BCR expression, leading in the worst case to Hodgkin's lymphoma (11, 13). In contrast, if a naïve B cell which has a functional BCR is newly infected and receives the survival signal, the additional signal of LMP2A might resemble in total an activated BCR, forcing EBV to lytic infection. In the presence of LMP2B, the signal of LMP2A would be downregulated, not leading to activated lytic EBV as was suggested previously for high levels of LMP2A (32).

In conclusion, the data presented here provide evidence that LMP2B is involved in the regulation of switching from latent to lytic EBV in B cells harboring functional EBV. Based on our present and previous findings for Akata cells (27) together with observations made for the EBV-negative cell line BJAB (29), we suggest that LMP2B has an impact on the activity of LMP2A, resulting in increased susceptibility to induction of lytic EBV infection through modulation of BCR and downstream signaling.

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